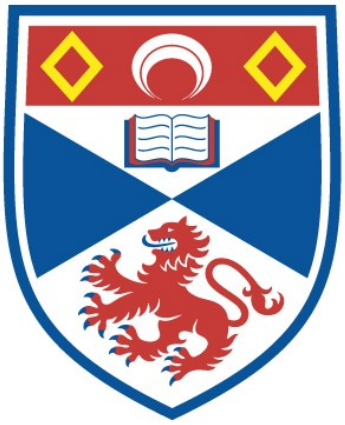
NON-LINEAR MEMBRANE PROPERTIES OF INSECT MOTORNEURONES

Julian C. Hancox

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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NON-LINEAR MEMBRANE PROPERTIES OF INSECT MOTONEURONES

A thesis submitted to the University of St. Andrews for the degree of Doctor of Philosophy

by

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Signed Date

23.04.91

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 in October of 1987 and as a candidate for the degree of Ph.D. in September of 1988.

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23.04.91

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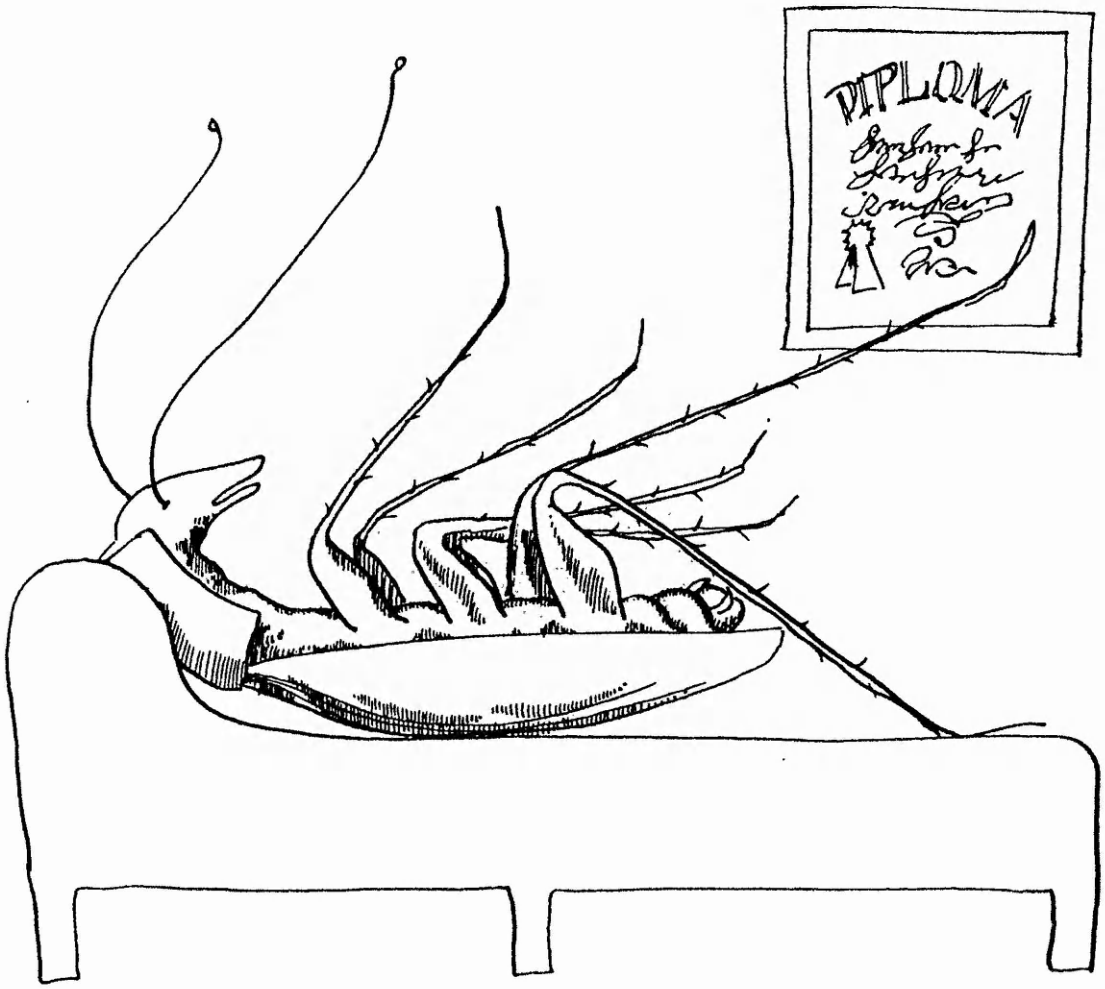
Finally, a special thanks to my parents for their support and encouragement throughout my education.

This thesis is dedicated to the late Joanna Copley.



Understanding your cockroach

Cartoon by Powell.
From: The cockroach combat manual. By A.M. Frishman and A. P. Schwartz. *Morrow quill paperbacks (N.Y.)*

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FIGURE INDEX

SUMMARY.

1. Electrical characteristics of the cell body of an identified motoneurone, the 'fast' coxal depressor motoneurone (D_f), from the cockroach (*Periplaneta americana*) have been studied under current- and voltage-clamp.

2. In response to low magnitude, relatively long duration depolarising current pulses, D_f could generate plateau potentials, regenerative events which often far outlived the duration of the applied depolarisation.

3. Plateau potentials constitute an inherent property of the neurone because they could be evoked in somata that had been surgically isolated from other parts of the neurone (the soma is devoid of synaptic contacts); these experiments also demonstrated that the soma of this neurone can participate in the generation of plateau potentials.

4. Plateau potentials were often surmounted by attenuated action potentials; these correlated 1:1 with axonal impulses recorded extracellularly from the axon of the neuron.

5. Plateau potentials were associated with an increase in membrane conductance. Under voltage-clamp, cells which exhibited plateau potentials possessed a region of negative slope resistance in their current-voltage relationship.

6. Plateau potentials in D_f were observed to be calcium-dependent. A series of current- and voltage-clamp experiments indicated that the calcium channels involved in plateau potential production differ from those which can mediate calcium-dependent action potentials following pharmacological treatment of this neurone.

7. Plateau potential production in D_f was suppressed by the application of GABA (10^{-4}M). Spontaneous plateau potentials could be recorded following application of picrotoxin (10^{-5}M) or pentylenetetrazole (25mM).

8. Recordings taken from two other 'fast' motoneurones, cell 3 (from the cockroach) and FETi (from the locust, *Schistocerca gregaria*) indicated that the ability to generate plateau potentials may not be restricted to D_f.

9. Although freshly dissected, recently impaled neurones responded to relatively brief depolarising current pulses with a series of graded, damped membrane oscillations, the excitability of many preparations increased with time from dissection: many cells became able to generate all-or-none action potentials in response to such pulses (these differed from the attenuated axonal spikes which often surmounted plateau potentials). The appearance of these events did not correlate with consistent changes to the resting potential or input resistance of neurones.

10. Time-dependent action potentials were calcium-dependent and could be recorded from 'intact' cells and isolated neurone somata. These action potentials could also co-exist with plateau potentials; such co-existence provides evidence for different classes of calcium channel in untreated insect neurones.

PREFACE.

Insects have provided a number of preparations with which neurobiologists have addressed questions in a variety of areas, ranging from receptor pharmacology to the neural bases of behaviour. Why should these animals receive significant attention at all? At the time of writing, the ability to confront such an issue is essential, for experimental scientists are forced to justify their existence now, more than at any time previously.

One fundamental reason for the scientific interest which insects have aroused is that they constitute the most successful class in the animal kingdom, making up more than ten times the number of species than that of the rest of the animal kingdom put together. It is hardly surprising, therefore, that such a successful order has profound ecological and socio-economic significance. On the one hand, insects serve a useful purpose as pollinators: two thirds of all flowering plants depend on insects for pollination, including a number of economically important food crops. Man has taken advantage of this in placing colonies of bees near fruit orchards and fields of commercially viable crops. On the other hand, insects have an awesome destructive capacity: they defoliate forests, attack timber buildings, and spoil crops and stored food. In the extreme, the mass destruction of crops by swarms

of locusts and grasshoppers results in famine, sickness and sometimes death. This destructive capacity, together with the fact that insects transmit a number of diseases to man (such as yellow fever, sleeping sickness, typhoid fever, dysentery and, perhaps most significant of all, malaria) means that there is considerable incentive to limit the deleterious effects of this order on man and his environment.

As a consequence, of the money spent on insect research, much is directed towards insecticide development. Such work is frought with problems: insecticides must target insect pests whilst leaving the rest of the food chain unaffected and the rapid evolution of insecticide-resistant species demands newer, more effective insecticides. Clearly, novel insecticides depend on either novel sites or modes of action, which in turn demand research into insect physiology in general, and respiratory and neural systems in particular.

However, insects merit attention for other reasons than simply finding ways to control them; we can also increase our understanding of more general physiological principles by studying these animals. In 1948 Sir Vincent Wigglesworth said "Insects are so varied in form, so rich in species and adapted to such diverse conditions of life that they afford unrivalled

opportunities of physiological study." Certainly, the insect nervous system has provided neurobiologists with attractive experimental models. Compared to the large - and correspondingly more complex - central nervous system of higher vertebrates, the insect nervous system is relatively simple (at least in terms of the actual numbers of neurones which comprise it). It also contains many large and functionally identified neurones which are easily accessible and therefore lend themselves to electrophysiological study. In addition, of course, the moral dilemma facing workers using 'lower' animals is arguably some degree smaller than that facing those working on higher vertebrates (which in many cases are inappropriate to answer questions which can really only be addressed using less complex experimental models).

Perhaps there are no better insect nervous systems with which to work than those of the locust, *Schistocerca gregaria* or *Schistocerca nitens*, and the cockroach, *Periplaneta americana*, not simply because these species are particularly large and therefore relatively easy to work on, but also because locusts and cockroaches present themselves as such formidable adversaries to *Homo sapiens*. Locusts, basking in the notoriety afforded them by biblical scholars, proliferate in arid climates. Cockroaches, on the other hand, are so adaptable that they can, and do,

live in any number of vastly differing environments, often in association with man.

Cockroaches pre-date and yet have out-survived dinosaurs and fossil remains indicate that there have been few structural changes. These hardy animals are remarkably resistant to a wide range of insecticides and to radiation, and can utilise numberless food sources. These factors, combined with their prolific reproductive capabilities means that, as pests, they are virtually impossible to eradicate, and as such deserve some respect. Present day cockroaches belong to the group *Paleoblattidae* of which four species exist as household pests in America, Europe and the Orient. It is thought that these four major species of cockroach all originate from Africa. Some, including the so-called American cockroach, travelled across the Atlantic in slave ships. It is the nervous system of this animal which provides the central focus for this study.

CHAPTER 1. GENERAL INTRODUCTION

This thesis is concerned with identifying and characterising non-linear membrane properties of insect motoneurones. Neurones can demonstrate a variety of non-linear membrane properties; examples include voltage-dependent membrane rectification, all-or-none action potentials and burst-forming potentials. This introductory section aims to provide a general background to certain non-linear membrane properties (bursting activity and plateau or driver potentials) which are relevant to the bulk of the work presented here. It also provides background information on the current knowledge of insect motoneurone properties, to aid the reader in placing the current work in context.

1.1. Rhythmic pattern generation and the biophysical properties of membranes - a brief historical perspective

This first section considers the discovery of neurones with intrinsic burst-forming properties from a historical perspective. By taking selected examples, it also explores the parallel development of concepts in the fields of membrane biophysics and neural networks.

Julius Bernstein was perhaps the pioneer of modern bioelectric research. At the turn of the century (1902) Bernstein formulated his 'membrane hypothesis': cells possessed a selectively permeable membrane with positive charge on the outside and negative charge on the inside.

However, it wasn't until much later that significant developments were made in terms of electrophysiological recordings. Notably, Hodgkin and Huxley (1939; 1945) and Curtis and Cole (1940; 1942) recorded action potentials in squid axons using intracellular electrodes. Later, Hodgkin and Katz (1949) formulated and confirmed their 'sodium hypothesis' which attributed the action potential overshoot to a switch in selective permeability of the axon to external Na^+ which entered the cell during the action potential.

At about the same time Marmot and Cole (1949) independently developed the technique of voltage-clamp, which revolutionised the way in which membrane currents could be investigated. The technique was further developed and utilised to great effect by Hodgkin, Huxley and Katz (1952) whose work laid the foundations for our current knowledge of membrane bioelectric behaviour, and determined the direction subsequent work was to take. In broad terms, the voltage-clamp technique, using two intracellular electrodes, involved controlling the voltage across a cell membrane by a negative feedback circuit and

directly measuring the resultant ionic movements as small transmembrane currents.

Hodgkin and Huxley (1952a-d) determined that two ion types were essentially responsible for excitability in the squid giant axon: sodium ions were shown to carry the depolarising phase of the action potential and potassium ions to carry the repolarising phase. In numerically describing excitability they produced models essentially based on a two channel system, ascribing three gating variables to these two channel types: the sodium and potassium activation variables m and n and a sodium inactivation variable h.

The 1950s saw a rapid expansion in the number of preparations which were suitable for detailed electrophysiological investigation. This was largely due to the development of the glass microelectrode (Ling and Gerard, 1949) which meant that relatively small neurones became accessible for experimentation, since the electrode size was no longer such a technical limitation. Thus, the electrical properties of a range of preparations could be examined and compared to those of the squid giant axon.

The Hodgkin and Huxley model of excitability was given considerable weight by a series of experiments by Coombs, Eccles and Fatt (1955) using mammalian spinal motoneurones. These experiments, utilising conventional recording techniques (not voltage-clamp),

showed that the ionic basis for the electrical activity in these neurones was similar to that demonstrated by Hodgkin and Huxley in the squid giant axon. Thus, it appeared that mammalian neurones functioned in a similar way to the squid giant axon and, perhaps, that a Hodgkin-Huxley-type model of excitability could be universally applied.

Other work of the time did reveal that for systems other than the squid giant axon ions other than sodium could carry the upstroke of the action potential. For example, 'mixed-dependency' spikes, in which the spike amplitude depended on both external sodium and calcium ions, were recorded from frog spinal ganglion cells (Koketsu *et al.*, 1959) and purely calcium-dependent spikes were recorded from the giant muscle fibre of the barnacle (Hagiwara and Naka, 1964). None-the-less, descriptions of excitability could be based upon the Hodgkin-Huxley model and, to a large extent, this dominated thinking for some twenty years after their first experiments. In fact, the impact of the 'Hodgkin-Huxley' neurone has been far reaching; for it has affected concept development in a number of developing areas of research.

The advent of relatively sophisticated electrophysiological recording techniques allowed investigations of a number of interesting physiological questions in ways that had not been

possible previously. Some of these questions related to mechanisms underlying the generation of various rhythmic, or cyclical activities such as spontaneous rhythmicity in the heart, respiration, cyclical movements of the gut and internal organs and, of course, locomotion.

In 1959, Huxley demonstrated that the Hodgkin-Huxley equations could reproduce the repetitive firing of nerve in calcium-deficient solutions. This prompted studies on pacemaker activity in the heart by Noble, working with cardiac Purkinje fibres. In 1960 he was able to construct a Purkinje fibre pacemaker model as a development of the Hodgkin-Huxley equations, using some of his early studies on cardiac muscle as the experimental bases for his model. This model was based on the decay of a potassium conductance (g_k) activated during the action potential. The decay in outwardly directed (hyperpolarising) current was assumed to unmask a background leak current which drives the membrane potential gradually towards threshold for firing. This 'g_k model' appeared to satisfactorily account for rhythmic firing in cardiac cells and dominated the work on rhythmic firing in the heart for some fifteen years after its conception, until it became clear that part of the evidence supporting the model was fundamentally incorrect (DiFrancesco, 1981; for reviews see Noble, 1983, 1985). The point of significance here, however, is that for a long time

rhythmic firing in heart cells appeared to follow mechanisms which could be predicted from the Hodgkin-Huxley model.

It is not without reason, then, that neuroscientists addressing the problem of pattern generation in neural circuits assumed that individual neurones all essentially behaved as variants of the 'Hodgkin-Huxley' neurone. Furthermore, it is probably not unfair to cardiac physiologists to state that neuroscientists were relatively disadvantaged in addressing the mechanisms underlying oscillatory rhythm generation, since neural circuits are so much more complex in morphological terms than the heart, which is largely a functional syncytium (the inference being that it has never been difficult to think of cardiac rhythmicity as resulting from inherent membrane properties of individual cardiac cells, since all cells in the myocardium are likely to possess similar properties).

Cajal had elegantly demonstrated that the nervous system is composed of individual elements separated physically from each other, that these individual elements may be morphologically distinct, and that they interacted *via* specific contacts (1888, 1934). However, it was not intuitively obvious that neurones could be, to an extent, independent functional entities, and the available electrophysiological evidence pointed to similarities, rather than

differences in excitable properties for a variety of cellular types.

How, then, could such morphologically distinct, and yet apparently electrically similar, neurones interact to produce the rhythmic impulse patterns required for the generation of specific behaviours such as walking and chewing? The most obvious solution lay in the contacts between the neurones which formed the network responsible for the behaviour. Therefore, early models of rhythm generation reproduced rhythmic impulse outputs purely on the basis of the specific connections between neurones: the networked output was a result of synaptic connectivity between neurones with essentially similar electrical properties.

The simplest network which is theoretically capable of demonstrating oscillatory activity is the 'half-centre' oscillator (Brown, 1914). This requires that two neurones are connected by reciprocally inhibitory synapses, and that some process intrinsic to each neurone causes the inhibitory effect of each neurone upon the other to lessen with time following activation of its activity (for example synaptic fatigue and accommodation). Tonic excitation from an external source would make the two reciprocally inhibitory neurones fire action potentials in alternating bursts. Model networks based upon reciprocal inhibition abounded in the 1960s, and the new craze for computer-modelling led to the

development of 'neuromimes', none of which worked too well, since, whilst they could replicate alternate firing patterns, accommodation and synaptic fatigue proved harder to model.

By the mid 1960s, however, it was becoming apparent that not all neurones behaved in ways that could be predicted by the Hodgkin-Huxley equations. Studies on the abdominal ganglion from the Sea-Hare, *Aplysia*, had revealed a neurone, cell R_{15}, which in isolated ganglia produced a distinct electrical rhythm: bursts of action potentials separated by periods of hyperpolarisation (Strumwasser, 1965). The interburst hyperpolarisation was observed to give way to a slow membrane potential depolarisation which eventually takes the cell above threshold for initiation of the next burst. During the burst, the action potentials were observed to first increase in frequency, attain a maximum rate and then decline in frequency towards the end of the burst ('parabolic' bursting). It was apparent that this repetitive bursting activity was not attributable to synaptic modulation of the neurone, since the rhythm persists even when the cell has been totally isolated (Alving, 1968). Therefore, cyclical bursting activity in this cell could only result from membrane properties endogenous to this individual neurone; these would necessarily be somewhat more complex than those of the simple Hodgkin-Huxley model. A subsequent voltage-clamp

study on rhythmic firing in gastropod nerve cells showed that, at the least, one additional conductance must be involved in repetitive firing to those present in the Hodgkin-Huxley model (Connor and Stevens, 1971). A current, activated by depolarisations from relatively negative potentials, slowed the approach of membrane potential towards the threshold for action potential production. This transient outward current, I_A, is activated by the afterhyperpolarisation following action potentials, and determines the interval between successive action potentials.

The discovery of endogenously bursting, or 'oscillator' neurones rather divided opinion as to the mechanisms of motor pattern generation; some held the belief that synaptic connections among neurones (none of which required unusual membrane properties) primarily determined the pattern, whilst others began to invoke a single neurone with special membrane properties as setting the rhythm. These hypotheses became known as 'network' and 'oscillator' hypotheses.

The case for networked activity patterns was strengthened by the incorporation of the phenomenon of postinhibitory rebound (PIR) into the 'half-centre' model by Perkel and Mulloney in 1974. PIR had been observed in the early 1960s in neurones from a number of animals including the toad (Fumikami, 1962) and cat (Kandel and Spencer, 1961). PIR describes the

transient overshoot of membrane potential above the initial resting potential following release from inhibition (it is analogous to the anode-break response described for the squid giant axon by Hodgkin and Huxley in 1952d). By combining reciprocal inhibition and PIR, antagonistic neurones could generate stable motor patterns, since PIR could serve the same purpose as accommodation and synaptic fatigue in this model. Perkel and Mulloney concluded that in particular cases 'an experimental search for driving oscillator neurones may be fruitless'.

In contrast, experimental evidence was beginning to indicate that oscillator and network hypotheses were not mutually exclusive.

Much of this evidence came from work on the stomatogastric ganglion (STG) of the lobster (*Panulirus interruptus*). With relatively few neurones, it appeared to offer a system with which it might be feasible to uncover the mechanisms responsible for rhythmic activity. The thirty or so neurones which comprise the STG produce two distinct rhythms underlying different digestive behaviours of the stomach (Maynard, 1972). The slow gastric rhythm appeared to result from a network of synapses between 12 neurones which were 'passive', possessing only simple capabilities (repetitive firing, for example) (Mulloney and Selverston, 1974; Selverston and Mulloney, 1974). However, the faster pyloric rhythm

seemed to be more complex: a separate group of three endogenous oscillators synaptically controlled 11 follower neurones, which again appeared to possess only simple firing characteristics (Maynard, 1972; Maynard and Selverston, 1975).

The picture which emerged from these studies was that bursting activity could arise from one of two mechanisms. Firstly, it could arise as a result of specific synaptic connections between neurones with simple firing properties, utilising properties such as reciprocal inhibition, repetitive firing and post-inhibitory-rebound. Secondly, it could result from special membrane properties which made neurones endogenously 'bursty'. In circuits such endogenous bursting neurones could interact with 'passive' (non-'bursty') follower neurones which faithfully responded to their synaptic inputs in an essentially linear manner. Under such circumstances, endogenous oscillators would essentially set the timing of the rhythm.

In 1978, a 're-examination' of the membrane properties of the passive neurones of the STG by Russell and Hartline revolutionised concepts in bursting pattern generation. They found that many 'passive' neurones actually possessed 'active' membrane properties which contributed to bursting. They observed that many STG neurones could generate 'plateau potentials',

prolonged regenerative depolarisations resulting from inherent membrane properties. These events resembled regenerative potentials which had been recorded from neurones from the cardiac ganglion of *Squilla oratoria* (Watanabe *at al*, 1967), and appeared similar to the depolarising portion of the activity recorded from spontaneously bursting molluscan and crustacean neurones. Plateau potentials could be elicited by relatively brief current pulses or synaptic excitation and could far outlast the duration of the excitatory drive. They were active, occurring above a distinct threshold, independent of the pattern of activity in presynaptic neurones (and therefore an intrinsic property of cells themselves); and they appeared to terminate spontaneously, but could also be prematurely terminated by applied hyperpolarisations. In addition, some cells appeared to lose the ability to generate plateaux if central input was abolished, suggesting that the expression of these events was susceptible to modulation.

The importance of this study cannot be overstated, since this work suggested that plateau potentials endowed follower neurones with the ability to respond in a *non-linear* fashion to their synaptic inputs: a relatively small, brief excitation could produce an active depolarisation which in turn could take the cell above the threshold for action potential production, resulting in a long duration impulse-burst. Prior to this finding, synaptic connectivity

had provided the main focus for work on pattern generation in neural networks. Now it appeared that the membrane properties of individual cellular components would require significant attention if pattern generation were to be fully understood, even in a circuit with relatively few constituent neurones such as the STG.

This, then, sets the historical context in which spontaneous bursting activity and plateau potentials came to light. Since the development of the above concepts, any investigation of the mechanisms underlying rhythmic pattern generation has necessarily included searches for both bursting neurones and neurones demonstrating plateau potentials.

A more detailed consideration of bursting potentials will now be given in terms of: their identification; prevalence; associated membrane properties; ionic mechanisms; and modulation.

1.2. Plateau potentials, bistability and 'burstiness'

In their original study, Russell and Hartline (1978) applied several criteria for defining plateau activity in neurones from the lobster stomatogastric system.

These criteria included: (a) the ability of the neurone to respond to a relatively brief depolarisation with an all-or-none transition between one (resting) potential and a more depolarised relatively stable region of potential; (b) that only currents above a certain threshold evoke such responses; (c) that in certain cases a neurone could produce bursts in the absence of patterned synaptic input.

From these criteria several plateau properties can be immediately inferred: that the events are voltage-dependent (exhibiting a threshold); that they are regenerative (outlasting the drive which evokes them) and that they are an endogenous neuronal property.

Selverston, Miller and Wadepuhl (1983) refer to plateaux as 'bistable' membrane potentials which are triggered by a short-duration excitation and can be terminated by a short hyperpolarisation. In this context, 'bistability' refers to two 'stable' levels of membrane potential: the resting potential (which is truly stable) and the semi-stable level of potential represented by the peak of the plateau-response. Hartline *et al* (1988) refer to 'key' criteria for

identification of plateau potentials in STG neurones as the ability to trigger all-or-none impulse-bursts with brief depolarising pulses and the ability to terminate bursts prematurely with brief hyperpolarising pulses. Perhaps one other criterion should be added: the events must be the result of membrane-properties intrinsic to the neurone from which they have been recorded.

It is important that a distinction be drawn between spontaneous bursting and burst forming capability. Both types of activity are the result of bistable membrane properties, and share (to a great extent) similar underlying mechanisms; yet they differ in one fundamental respect: the former refers to recurrent, rhythmic activity intrinsic to a neurone and characterized by depolarising, bursting potentials and an interburst pacemaker potential (such as the activity described for *Aplysia* R_{15} by Strumwasser); the latter refers to the capacity to respond to various types of input with a burst. Plateau potentials can be likened to the second phase of activity in endogenous bursters, which ensues once the bursting pacemaker potential has taken the membrane potential beyond the threshold for bursting activity (Selverston and Moulins, 1985).

It is also important to realise that bistable membrane properties (which probably underlie all endogenous

rhythmic cellular activity) are widespread. Bistability has been observed or inferred for neurones from many preparations. Examples include: molluscan burster neurones such as R_{15} (Strumwasser, 1965; Alving, 1968; for review see Adams and Benson 1985; 1989; and Benson and Adams, 1987); crustacean stomatogastric ganglion neurones (for review see Hartline *et al,* 1988); crustacean cardiac ganglion neurones, where the underlying regenerative event has been termed a 'driver' potential (Tazaki and Cooke, 1979a-c; for review see Cooke, 1988); neurones from the leech heart (Calabrese, 1979); motoneurones from the crayfish (Sillar and Elson, 1986), lamprey (Wallén and Grillner, 1987), cat (for review see Kiehn, 1991) and turtle (Hounsgaard and Kiehn, 1989). Oscillatory neuronal properties may play important roles in mammalian CNS function (reviews by Llinás 1988; 1989) and burst forming capabilities have been seen (for example) in Purkinje cell dendrites (Llinás and Sugimori, 1980); hippocampal pyramidal cells (Hablitz and Johnston, 1981); thalamic neurones (Deschênes *et al,* 1982; 1984) and even human neocortical neurones (Foehring and Wyler, 1990). In addition, bistable properties appear to mediate switching between two stable resting potential levels in cardiac Purkinje fibres (Gadsby and Cranefield, 1977), cyclic membrane conductances to be involved in slow waves of contraction in dog colonic muscle (BarajasLopez *et al,* 1989), and spontaneous bursts of contractions to be

recorded from guinea-pig ileum (Ito *et al*, 1988). Bistable properties may, therefore, be common among different excitable tissues which exhibit rhythmic activity.

1.3. Bistable membrane behaviour, the I-V relationship and negative resistance.

Fundamental to our current understanding of burst-forming mechanisms lies the observation, from voltage-clamp experiments, that bursting neurones possess in their current-voltage (I-V) relationship a region of negative slope conductance or, by tradition, negative slope resistance (NSR) (Fig. 1.1). This region of NSR occurs at more negative membrane potentials and is associated with currents exhibiting a slower time course than negative resistance properties associated with action potential production.

Voltage-clamp experiments on cell R_{15} of *Aplysia* (Wilson and Wachtel, 1974) followed the observation that, under current-clamp, it is impossible to stabilize the membrane potential of bursting neurones between the extremes of the cycle of oscillation. By clamping the membrane potential of this cell at the least negative point in the cycle of oscillation and applying long-duration hyperpolarising command steps, it was possible to view 'steady-state' currents (currents which showed only slow inactivation, if any) in the absence of complicating fast currents (which would be inactivated during such a protocol). Cells exhibited a positive slope resistance at relatively negative potentials, but at more positive potentials (approximately -60mV to -20mV), corresponding to the voltage-range where oscillatory behaviour occurs, a

distinct region of negative-resistance was observed. If cells were cooled from 22° to 10°C bursting activity ceased, and the current voltage relationship became linear. It seemed that the negative resistance characteristic (NRC) was associated with bursting behaviour. This was supported by the observation that non-bursting neurones at 10°C became bursters at 22°C and took on a NRC. A related study (David, Wilson and Escueta, 1974) showed that normally silent neurones from *Aplysia* which became bursting in the presence of the convulsant drug pentylenetetrazole, did so as a result of the induction of a region of NSR in the I-V relationship, the magnitude of which appeared dose-dependent. The importance of the NSR in the I-V curve was further highlighted by experiments performed upon bursting cells from the molluscs *Otala lactea* and *Aplysia* (Smith, Barker and Gainer, 1975) and *Helix pomatia* (Eckert and Lux, 1975) which indicated that the region of NSR was associated with a non-inactivating inward current. Later experiments demonstrated a NRC in motoneurones from the anaesthetized cat (Schwindt and Crill, 1977), which have subsequently been observed to produce plateau potentials in the decerebrate animal. (Hounsgaard et al, 1988). Bistability of cardiac Purkinje cells has also been associated with a region of NSR in the current-voltage relationship.

An additional feature of spontaneously bursting neurones is that the I-V relationship crosses the zero-current axis, or abscissa, only at relatively positive voltage; at negative voltages it lies below the abscissa, thereby producing a continuous, depolarising, flow of current (see Fig. 1.1A). Under current-clamp, a cell with such an I-V curve will not possess a steady resting potential, but will tend to fire action potentials in repetitive bursts (Benson and Adams, 1987).

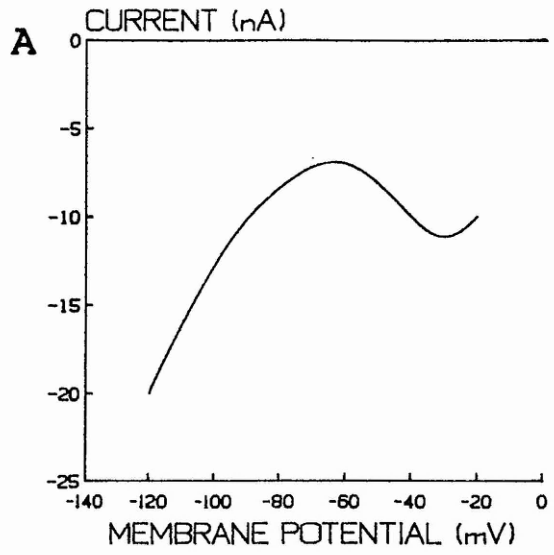
If, on the other hand, the I-V relationship crosses the zero-current axis at negative voltage with positive slope then the intercept-voltage will form a point of stability (provided the intercept is negative to the threshold for action potential production). The NRC of cardiac Purkinje fibres, for example, allows the I-V curve to cross the abscissa with positive slope at two potentials, thereby accounting for the two stable resting potential levels observed for these fibres (Gadsby and Cranefield, 1977; see also Fig 1.1B).

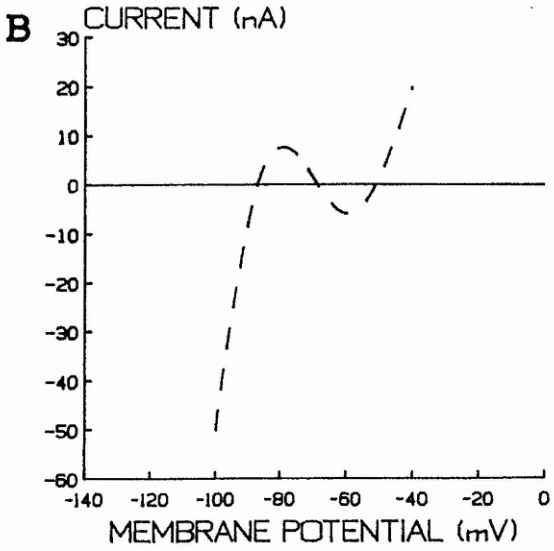
In neurones with burst forming capability, but which are not spontaneously bursting, the I-V curve may lie positive to the zero current axis at all potentials and the region of NSR may or may not intercept the abscissa (Schwindt and Crill, 1977; see also Fig 1.1C). In such cases the cell will not burst without some form of excitation; but any influence

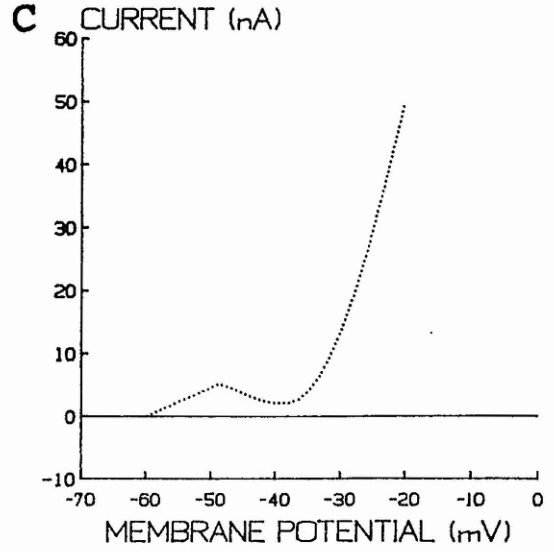
that shifts the membrane potential into the NSR range will allow the cell to respond regeneratively.

FIGURE 1.1: I-V relationships representing different properties of cells possessing a negative resistance characteristic. (A) A 'typical' I-V curve for a molluscan spontaneously bursting neurone. (B) I-V relationship for a cell-type with two stable values of resting potential (cardiac Purkinje fibre); potentials where the I-V curve transects the voltage axis with positive slope represent points of relative stability. (C) For other cell-types (some motoneurones, for example) the direction of current flow is outward at potentials which lie positive to the normal resting potential; however, a region of negative slope in the I-V relationship permits regenerative activity over certain potential ranges (such ranges are distinct from those which underlie action potential generation).

Curves (A-C) do not represent original data but are representations of data drawn from a variety of sources: (A) - Adams and Benson, 1985; Benson and Adams, 1987; (B) - Gadsby and Cranefield, 1977; (C) - Schwindt and Crill, 1977.


A
CURRENT (nA)
0
-5
-10
-15
-20
-25
-140 -120 -100 -80 -60 -40 -20 0
MEMBRANE POTENTIAL (mV)


B
CURRENT (nA)
30
20
10
0
-10
-20
-30
-40
-50
-60
-140 -120 -100 -80 -60 -40 -20 0
MEMBRANE POTENTIAL (mV)


C
CURRENT (nA)
60
50
40
30
20
10
0
-10
-70 -60 -50 -40 -30 -20 -10 0
MEMBRANE POTENTIAL (mV)

1.4. The ionic basis of burst formation.

Although the patterns of axonal activity generated by bursting neurones, whether crustacean, molluscan or vertebrate, may appear qualitatively similar, the ionic mechanisms underlying bursting activity differ considerably. Because of this, it is not possible to describe a universal model, invoking similar ion conductances, which can produce bursting activity. Rather, it is convenient to consider separately the origin of different types of bursting activity.

1.4.1. Molluscan bursting neurones

Without doubt, the bulk of our knowledge on bursting mechanisms comes from work on molluscan bursting neurones, particularly cell R_{15} of *Aplysia*, which according to Adams and Benson (1985) is probably the most studied neurone after the squid giant axon. Bursting in R_{15} is of the form shown in Fig. 1.2A.

On theoretical grounds, bursting in molluscan cells requires a minimum of three different currents: a depolarising current, I_{NSR}, is needed to initiate the burst; an activity-activated hyperpolarising current I_H is needed to terminate the burst; and a regenerative excitatory current is required so that the burst is not terminated by I_H after the first action potential (Adams and Benson, 1989). The reality is more complex, since it appears that

currents may not be easily resolved into such theoretical components and are likely to include a variety of ionic mechanisms.

1.4.1.A. Burst initiation

The maintenance of endogenous bursting activity requires a continual inward driving force (Smith, Barker and Gainer, 1975). This could be supplied by a simple inwardly directed leakage current, which combines with cyclical changes in outward current to generate bursting. However, in some instances leakage currents for endogenous bursters are outwardly directed (for example, Eckert and Lux, 1975). Cyclical changes in outward current may play a part in generating the inward drive for bursting in some neurones, however (Wilson and Wachtel, 1974; Smith, Barker and Gainer, 1975). For example, study of an identified bursting interneurone from *Helix* suggests that a slow, persistent decrease in resting potassium conductance caused by firing of the cell acts to increase effectiveness of the burst-generating mechanism (Pin and Gola, 1987).

A second possibility is that part of the drive originates from an anomalously rectifying potassium conductance (Katz, 1949) which combines with I_{NSR} to drive the burst. In R_{15}, the potassium current which carries the bulk of the membrane current at highly negative potentials (in excess of -80 mV) demonstrates

inward rectification; it can carry substantial inward current, but only small outward current (see Benson and Adams, 1987). Thus, this conductance can provide an inward driving force which permits the membrane potential to enter the activation range for I_{NSR}, the current which initiates the regenerative depolarising phase of the burst cycle.

I_{NSR} is the current responsible for the negative slope region of the I-V curve. This current appears to have two components: a fast component (present in bursting and non-bursting neurones) and a slow component (restricted to bursting neurones) (see Adams and Benson, 1985). Initial evidence suggested that for *Aplysia* neurone R_{15}, I_{NSR} was attributable to a slowly developing inward current carried by sodium ions and mediated through an incompletely inactivating sodium conductance (Smith, Barker and Gainer, 1975). Later evidence, however, suggested that the NSR is abolished by removal of extracellular Ca^{2+} (Gorman *et al*, 1982), suggesting that the slow inward current was calcium-dependent; whilst the NSR for neurones L_2-L_6 has been described as sensitive to both sodium and calcium ions (Johnston, 1976). Benson and Adams (1985), however, report abnormal changes in membrane resistance following removal of either sodium or calcium ions from the bathing solution, which suggests that the changes in membrane properties observed during ion-

substitution experiments on *Aplysia* neurones may be the result of pathological changes to cells.

Eckert and Lux (1975) eliminated the NSR in I-V curves from *Helix* neurones using calcium-free saline and suggested a role for a slowly inactivating calcium conductance. Heyer and Lux (1976) found that the slow inward current from *Helix* neurones occurs simultaneously with potassium efflux; supporting calcium influx coupled to a calcium-dependent potassium efflux (Meech and Standen, 1975).

The slowly activating inward Ca^{2+} current described by Eckert and Lux (1975) shows extremely slow decay, and does appear to resemble the slow component of I_{NSR} in R_{1S}. Such a conductance has also been identified with a slow, calcium-dependent tail-current, I_B, recorded from some molluscan bursters (Adams *et al*, 1980; Smith and Thompson, 1987). For R_{1S}, the evidence is that this current contributes to I_{NSR}, although, in some neurones I_B has been described as synonymous with another current I_D, which is quite distinct from I_{NSR} and appears to be involved in burst maintenance (see below).

In summary, I_{NSR} is a relatively slowly activating current, in which activation is incomplete, slow, or completely absent. Its ionic dependence is unclear, since in different preparations, evidence indicates that it may be carried by sodium ions, calcium ions or a combination of both of these; it nonetheless has

pivotal importance to bursting activity, since the NSR region of I-V curves is essential for bursting activity.

1.4.1.B. Burst maintenance

The current which is responsible for burst maintenance in many molluscan neurones is the 'transient, depolarising after-current' (I_D). I_D has been described for *Aplysia* neurones R_{15} (Lewis, 1984) and cells L2-L6 (Kramer and Zucker, 1985a). I_D drives the impulse burst after the first action potential. It is incrementally activated by successive action potentials, maintaining depolarisation of the cell membrane and thereby the impulse burst, and accounts for the depolarising after-potential which follows the last action potential of each burst. I_D is an inward current (between -20mV and -120mV; Adams and Levitan, 1985), with slow kinetics (inactivating over hundreds of milliseconds). It appears to be calcium-dependent, since it is activated by calcium ion injection (Kramer and Zucker, 1985a) and blocked by calcium antagonists. Lewis (1984) suggested that I_D in R_{15} is probably activated by increased intracellular calcium carried by the slow inward current. Kramer and Zucker (1985a), working on left upper-quadrant bursters (LUQBs), found that I_D was sensitive to external sodium concentration but not to agents which blocked sodium channels (TTX), sodium/calcium exchange (ionic

substitution of lithium for sodium), or sodium/potassium exchange (ouabain). They suggested that this current is due to calcium-dependent activation of a non-specific cation conductance, and that I_D is probably distinct from I_{NSR}. More recent work (Lando and Zucker, 1989) has shown a linear dependence on intracellular calcium concentration of calcium activated non-specific cation currents from bursting *Apylsia* neurones.

There appear to be differences in I_D between R_{15} and the left upper quadrant bursters of *Aplysia*. For R_{15}, I_D can be recorded from neurone somata joined to their axons, but appears to be absent from cell bodies separated from the impulse-generating portion of the axon (Adams, 1985; Kramer and Zucker, 1985a). This does not appear to be the case for LUQBs (Kramer and Zucker, 1985a). Therefore, in the former case the current appears to be activated in response to an axonal action potential (Adams and Levitan, 1985), whereas such axonal involvement is not necessary for LUQBs (Kramer and Zucker, 1985a). I_B, a current in bursting pacemaker neurones from *Tritonia* (Smith and Thompson, 1987) which appears to perform a similar function to I_D in LUQBs from *Aplysia*, also appears to originate from the neurone soma.

In summary, I_D (or analagous currents, such as I_B from *Tritonia*) is responsible for maintaining the burst after the first action potential of the burst-series.

It causes a depolarising afterpotential following each spike, thereby maintaining the depolarised phase of pacemaker activity, and forms the depolarising afterpotential seen at the end of each burst cycle for molluscan bursters.

1.4.1.C. Burst termination

Two alternative mechanisms have been postulated to drive burst termination in molluscan bursting cells: firstly, activation of I_C, a calcium-dependent potassium current; secondly, activation of a 'transient hyperpolarising after-current' (I_H), possibly resulting from calcium-dependent inactivation of the background steady-state (slowly-inactivating) calcium current.

Evidence from a number of studies of the 1970s suggested that for R_{15} a rise in intracellular calcium throughout the burst activated potassium efflux from the neurone, resulting in the membrane hyperpolarisation Some of the this evidence was as follows: intracellular calcium injection increases potassium conductance in R_{15} (Meech, 1972), and direct measurements of intracellular ionized calcium using the dye Arsenazo III showed an increase in calcium during the burst (Thomas and Gorman, 1977; Gorman and Thomas, 1978). Therefore, a calcium-dependent potassium efflux (I_C; confirmed for *Helix* neurones in

1975 (Meech and Standen)) was proposed to account for these experimental observations. During bursting activity, the burst would persist until the outward driving force (resulting from I_C) exceeded the inward drive generated by the slow inward current (at this time I_D was unknown). At the point where the net driving force became outward the membrane would hyperpolarise. The intracellular calcium concentration would then slowly return to its resting level (since there would be no inward current) and the cell would gradually depolarise.

Although I_C can theoretically account for burst termination, current models of bursting for R_{15} do not depend on I_C, but instead invoke the transient hyperpolarising after-current, I_H, as leading to burst cessation (Adams and Benson, 1989). I_H was first reported for R_{15} by Lewis (1984) and subsequently verified for this neurone (Adams, 1985) and LUQBs of *Aplysia* (Kramer and Zucker, 1985b). Similarly to I_D, I_H is an activity activated current and may result from inactivation of a steady-state calcium current by transmembrane calcium fluxes during action potentials of the burst (Adams and Levitan, 1985). I_H demonstrates slower kinetics than I_D, however, peaking later and inactivating more slowly than I_D (Adams, 1985). Between -20mV and -120 mV this current is outwardly directed (Adams and Levitan, 1985; Kramer and Zucker, 1985b) and it appears to be

calcium-dependent (Lewis, 1984; Adams and Levitan, 1985; Kramer and Zucker, 1985b). The potassium-dependence of the current is unclear but it does not reverse at E_K (Adams and Levitan, 1985; Kramer and Zucker, 1985b). Similarly, there is evidence suggesting that I_H is independent of external sodium concentration (Adams and Levitan, 1985); it has been suggested that the loss of I_H following prolonged exposure to zero sodium observed in some studies (Lewis, 1984; Adams and Levitan, 1985 and Kramer and Zucker, 1985b) may be due to non-specific or harmful effects resulting from removal of this ion (Adams and Benson, 1985).

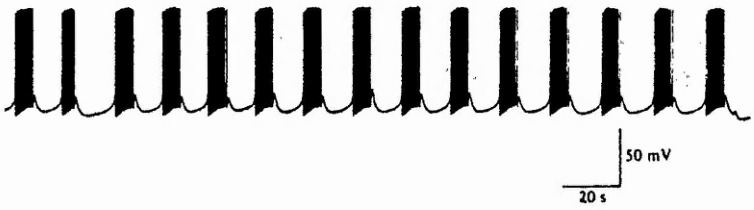
I_H can account for many experimental observations attributable to I_C, whilst accounting for properties of bursting in R_{15} that I_C cannot explain (this is discussed fully by Adams and Levitan, 1985 and Adams and Benson, 1985). Notably, Adams and Levitan (1985) could not find evidence for activation of a long-lasting K^+ current in normally bursting cells or following a series of depolarizing pulses (I_H does not actually reverse at E_K and appears insensitive to changes in external potassium concentration and to TEA application).

FIGURE 1.2: Bursting in *Aplysia* R15. (A) Recording of a series of spontaneous action potential bursts from R_{15} (Ai) and of a single action potential burst (Aii). Bursting consists of a gradual depolarisation to the threshold for action potential production followed by a series of action potentials. The last action potential of the series is followed by a depolarising after potential (marked in Aii with an asterisk) and an afterhyperpolarisation. (B) A simplified model of bursting in R_{15}, showing the interaction of the transient inward current (I_D) and transient outward current (I_B) in structuring the net membrane current (I memb) which underlies the action potential burst (top trace).

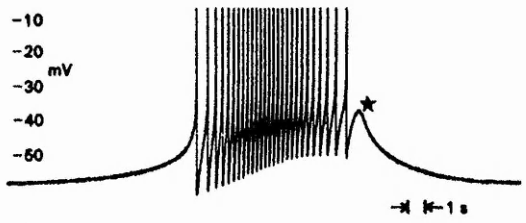
This figure does not contain original data. Information has been included from the following sources: (Ai) - Levitan *et al*, 1979; (Aii-B) - Adams, 1985.

A

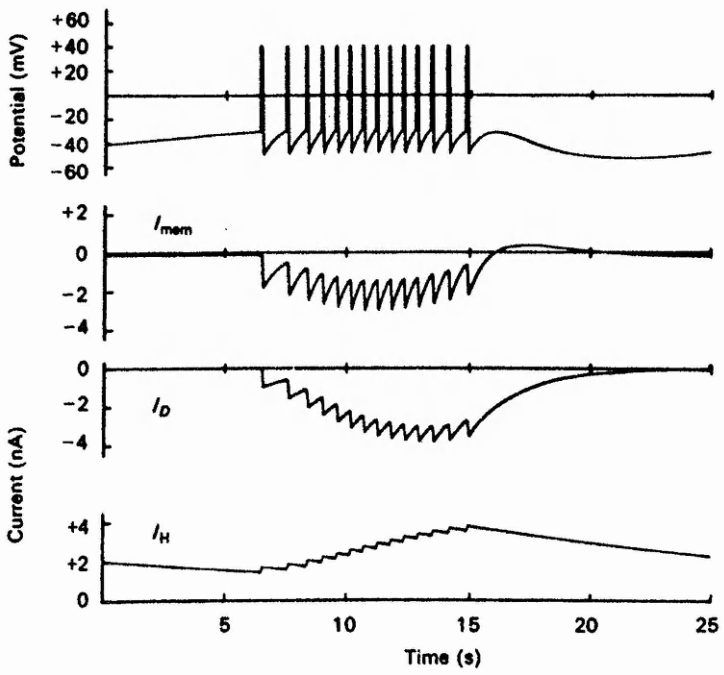
i


50 mV
20 s

ii


-10
-20
-30
mV
-40
-60
1 s

B


Potential (mV)
+60
+40
+20
-20
-40
-60
+2
0
-2
-4
I_{mem}
0
-2
-4
I_D
Current (nA)
+4
+2
0
I_H
5
10
15
20
25
Time (s)

1.4.1.D. The burst-cycle.

The activation of I_H is intimately connected to the activation of I_D and it is the interplay of these two currents which appears to determine burst formation for bursting neurones from *Aplysia* (Adams and Benson, 1989). Adams (1985) has even demonstrated that I_D and I_H alone could (in theory) generate bursting activity in R_{15}, provided some continual inward driving force were present (see Fig. 1.2B). As the neurone membrane potential approaches threshold for burst initiation, I_{NSR} (which provides much of the inward driving force) predominates. When the threshold is exceeded the first action potential of the burst is activated, which in turn activates I_D and I_H. The incremental activation of I_D initially exceeds that of I_H, making the net inward current greater than before the first action potential. This makes depolarisation towards the next action potential faster. This series of events is repeated with successive action potentials; I_D and I_H are incrementally activated to approximately the same extent by each action potential and each current summates temporally. However, the time-constant of temporal summation for I_H is much longer than that for I_D and it is this that eventually causes burst cessation. Once I_D has reached a maximum, its incremental activation with successive action potentials barely exceeds its level of deactivation following the previous action potential. I_H, on the

other hand, continues to increase throughout the burst, whilst the rate of depolarisation between successive action potentials becomes less. Eventually the outward drive of I_H exceeds the inward drive provided by I_{NSR} and I_D, and the cell hyperpolarises. This interplay between I_D and I_H can account for the 'parabolic' nature of the action potential bursts, reported for molluscan bursters such as R_{15} (Strumwasser, 1965), with spike frequency first increasing, attaining a maximum and subsequently decreasing towards the end of the burst. The rapid down-swing into the hyperpolarising phase of the cycle is not caused by sudden activation of an outward current, but by the rapid decay of I_D, which results from the relative rates of inactivation of I_D and I_H, and deactivation of I_{NSR}. During the interval between successive bursts I_H predominates. Depolarisation out of the PBH results from inactivation of I_H and this in turn activates I_{NSR}, which, due its regenerative nature, reinforces its activation (no doubt aided by the anomalously rectifying potassium conductance, I_R). In this respect, I_{NSR} may be considered to be the pacemaking current in R_{15}. Once the bursting threshold is reached the cycle of events starts again.

Adams and Benson have modelled bursting activity in R_{15} with much success, proposing the above combination of I_{NSR}, I_D, and I_H (1989). However, whilst taking into account a linear leak conductance, their model

does not account for I_R (which together with I_{NSR} accounts for anomalous rectification in R_{15}). Moreover components such as I_{NSR} and I_R have proved difficult to model in computer simulations of bursting (see Adams and Benson, 1985) and, therefore, inward driving forces have been combined to produce an artificial 'leak' conductance. In addition, whilst this model (or ultimately a variant taking into account additional conductances) may accurately reflect bursting in many molluscan neurones, it cannot be universally applicable. For example, in neurones from *Tritonia,* I_B maintains the depolarised phase of the burst cycle and produces the depolarising after-potential following individual spikes, whilst I_C activates more slowly but attains greater amplitudes than I_B (Smith and Thompson, 1987). In this case I_C appears to cause burst termination, the duration of PBH and may play a role in spike adaptation during the burst. Smith and Thompson, therefore, can account for bursting in *Tritonia* without postulating any slow hyperpolarising current such as I_H.

A summary table describing some of the main currents controlling bursting in molluscan neurones is given in Fig. 1.3.

CURRENT	IONIC DEPENDENCE	PROPOSED FUNCTION
I_R	Potassium	Inward driving force at highly negative potentials. (Fast kinetics)
I_{NSR}	?	Current responsible for negative slope region of I-V curve. Major source of inward drive to initiate burst. (Slow kinetics)
I_D (*Aplysia*) I_B (*Tritonia*)	Probably Ca-dependent cation conductance	Burst maintenance. Depolarising after-potential (Slow kinetics)
I_H	Possibly Ca-dependent inactivation of Ca current.	Burst termination and afterhyperpolarisation (Very slow kinetics)
I_C	Ca-dependent potassium	In *Aplysia* R_{15} and LUQBs unknown. Burst-termination and afterhyperpolarisation (*Tritonia*). (Intermediate kinetics)

FIGURE 1.3: Summary table of some of the major currents contributing to bursting in molluscan neurones. (Information obtained from sources cited in text).

1.4.2. Crustacean Bursting Neurones

Much of our understanding of the mechanisms underlying bursting activity in crustacean neurones comes from work on neurones from the stomatogastric system of the lobster (*Homarus americanus*) and cardiac ganglia of the lobster and crab (*Portunus sanguinolentus*). Both STG neurones (Russell and Hartline, 1978) and cardiac neurones (Tazaki and Cooke, 1979a) generate plateau potentials. In cardiac motoneurones from the crab (Tazaki and Cooke, 1979a-c; 1983a) and lobster (Tazaki and Cooke, 1983b) Tazaki and Cooke identified an event which they have termed a 'driver potential' since it appears to underlie plateau potential production. The driver potential, revealed by application of the sodium channel blocker tetrodotoxin (TTX) to *Homarus* and *Portunus* preparations, results from endogenous membrane properties which allow neurones to respond to a non-specific excitatory input with an impulse burst (Cooke, 1988). Driver potentials differ from plateau potentials since they are devoid of spiking activity and are of relatively short duration (on average 250 ms; Tazaki and Cooke, 1979b) whereas plateau potentials are of indefinite duration (Benson and Cooke, 1984). Whilst the latter can terminate spontaneously, in STG neurones repolarisation is often aided by inhibitory synaptic inputs (Hartline *et al* 1988). Benson and Cooke (1984) report that TTX resistant oscillations have been recorded from pyloric neurones of the STG

system under certain circumstances (for example: Raper, 1979) and Cooke (1988) believes that the driver potential is ubiquitous to neurones with rhythmic impulse activity. There are good reasons, then, for primarily considering driver potentials when discussing the ionic basis of rhythmicity in crustacean neurones.

Early ion-substitution experiments, performed on neurones from *Portunus* in the presence of TTX, indicated that driver potentials are carried by a voltage-dependent calcium current and that these events are limited by calcium-dependent outward currents (Tazaki and Cooke, 1979c). Subsequent experiments on neurones from the cardiac ganglion of *Homarus* have revealed substantially more information, both in terms of the localization and of the ionic mechanisms of driver potential production, since it has proved relatively easy to isolate neurone somata from *Homarus* for voltage-clamp experiments (Tazaki and Cooke, 1983b,c; 1986; 1990)

Using axonal ligatures on *Homarus* neurones, Tazaki and Cooke (1983b) were able to demonstrate that driver potential production was limited to the soma and proximal axon. This points to an important difference between bursting in crustacean and molluscan neurones. For some molluscan neurones, currents activated by action potentials appear essential in maintaining and terminating bursting

activity (see section 1.4.1). In contrast, since driver potentials can be recorded from a restricted portion of the cell which is separate from impulse-generating membrane (Tazaki and Cooke, 1983b,c), impulse activated currents cannot contribute to structuring driver potentials. (The same need not be the case for plateau potentials, since they receive at least some axonal input in the form of attenuated action potentials which surmount the peak of the response, and may involve sites other than the soma and proximal axon (Hartline *et al*, 1988; Cooke, 1988).

The distinct advantage of an isolated soma preparation over the intact neurone is that isolation allows a more detailed analysis of currents underlying the driver potential using voltage-clamp: fewer technical problems in 'space-clamping' are likely to be encountered by clamping a limited portion of the neurone. (In this respect cardiac neurones are superior to those from the STG since the site of plateau production in the latter may be electrically distant from the soma, making voltage-clamp analysis difficult (Hartline *et al* (1988)).

Voltage-clamp studies on ligatured neurons revealed three outward currents and a leakage current (I_L), the magnitude of which depended on the distance of the ligature from the soma (both are larger for cells with greater membrane area) (Tazaki and Cooke, 1983c).

TEA application blocks an outward current component analagous to I_K of molluscan neurones; under current-clamp TEA increases peak amplitude and duration of the response (Tazaki and Cooke, 1983c; 1986). 4AP enhances the rate of rise of the driver potential, but not its amplitude and duration; under voltage-clamp 4AP suppresses the early outward peak of current responses (Tazaki and Cooke, 1983c). This effect is most marked for clamps elicited from relatively hyperpolarised membrane potentials; thus this component has similar characteristics to the early outward current (I_A) described for molluscan neurones (Connor and Stevens, 1971).

A third outward current appears calcium-dependent (I_C); this was revealed by analysis of outward tail-currents (which reverse near E_K) which are smaller in the presence of the calcium antagonist Cd^{2+} (Tazaki and Cooke, 1983c). This outward current is resistant to TEA (Tazaki and Cooke, 1986).

It is likely that all three outward currents are involved in regulating bursting activity: TEA, 4AP and the calcium-dependent potassium channel blocker apamin have been reported to induce bursting activity in the isolated anterior burster (AB) neurone of the STG (Harris-Warrick and Johnson, 1985).

Voltage-clamp analysis has also revealed an inward current carried by calcium ions; this supports the evidence from current-clamp studies (Tazaki and Cooke,

1979c) that the driver potential is carried by calcium ions. The inward current (I_{Ca}) persists in TTX but is inhibited by Cd^{2+} and Mg^{2+} ions (Tazaki and Cooke, 1983c) and can by carried but not augmented by strontium and barium ions (Tazaki and Cooke, 1990). I_{Ca} appears to demonstrate considerable activation for small depolarisations (10mV) from potentials between -60mV and -50mV (Tazaki and Cooke, 1986). This potential range covers the range of normal resting potentials for these neurones and, therefore, the activation properties of I_{Ca} may reflect its pivotal role in generation of the driver potential, which has a threshold at approximately -40mV (Tazaki and Cooke, 1983c). I_{Ca} demonstrates maximum inward tail-current amplitudes for clamps to -10mV, declining for depolarisations positive to 0mV (Tazaki and Cooke, 1986; 1983c). Additional information suggests that I_{Ca} approaches a saturating value for steps to -5mV and more depolarised, and that the time required to attain peak I_{Ca} is voltage-dependent (Tazaki and Cooke, 1990). Whilst activating rapidly, I_{Ca} demonstrates slow inactivation (Tazaki and Cooke, 1983c, 1986, 1990), but this does occur during sustained depolarisation (Tazaki and Cooke, 1983c). However, inactivation appears to be dependent upon calcium fluxes, rather than upon voltage; inactivation is reduced when Ba^{2+} or Sr^{2+} replace calcium in the saline or in neurones injected with the calcium-chelator EGTA (Tazaki and Cooke, 1990). The recovery

of I_{Ca} from such inactivation takes several seconds, reflected in current-clamp experiments in which the amplitude of driver potentials can be reduced by repetitive stimulation at high frequencies (Tazaki and Cooke, 1979b; 1990).

By combining the outward and inward currents observed under voltage-clamp, it is possible to postulate the series of events which cause burst generation in cardiac neurones. Starting at the end of the driver potential, the membrane potential is hyperpolarised (similarly to molluscan bursters following each burst cycle). This hyperpolarisation results from a high potassium conductance (primarily due to I_C), rather than from the combined effects of inward current inactivation and activation of a slow outward current analagous to I_H, as in many molluscan bursters. The high outward conductance conducts incoming excitatory events only poorly. In addition, since I_{Ca} induces a relatively hyperpolarised membrane potential, incoming depolarising events at this time are likely to activate the fast transient K^+ current, I_A, which is most responsive at highly negative potentials. This combination of influences, together with the fact that the slow calcium current can only recover from inactivation gradually, reduce the excitability of the neurone to incoming excitatory input, and hold the membrane potential negative to the threshold for

driver potential production for some time. This ensures the interburst interval.

As inactivation of the slow inward current fades and I_C decreases due to the gradual fall in intracellular calcium concentration, the depolarising 'leak' current (I_L) moves the membrane potential into the range for activation for I_{Ca}. In neurones which are spontaneously active, I_L alone may suffice to depolarise the cell into the threshold voltage range for another driver potential (Benson and Cooke, 1984); in other neurones the combination of I_L with excitatory input eventually takes the neurone suprathreshold for another driver potential. The activation of I_{Ca} drives the depolarising phase of the driver potential. As the neurone depolarises, however, the voltage-dependent I_K is activated and the influx of calcium ions activates I_C. Once the sum of I_K and I_C exceeds I_{Ca} the net outward current terminates the event and the neurone hyperpolarises. Due to the slow inactivation kinetics of I_{Ca}, calcium influx and consequently intracellular calcium concentration remain high and I_C persists for some time.

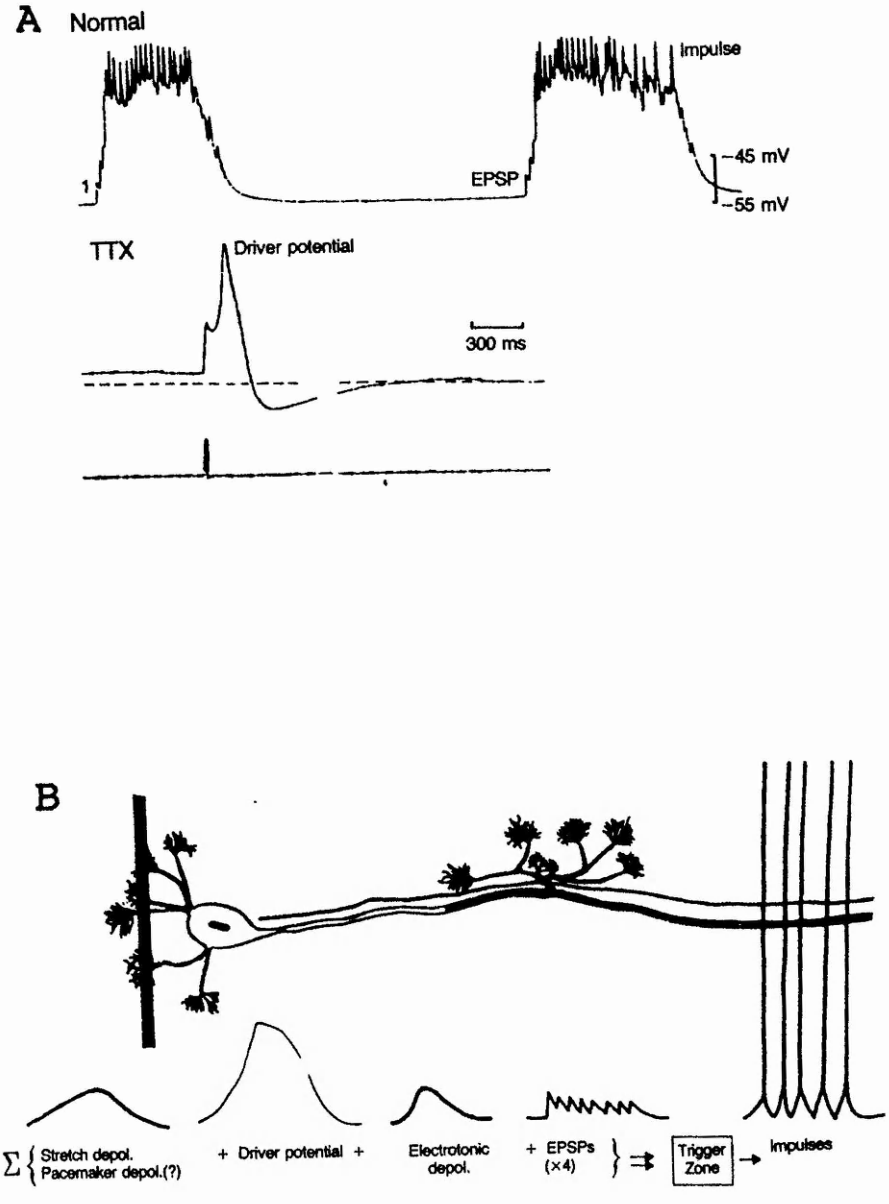
Once elicited, the driver potential can spread electrotonically from the soma and proximal axon to the distal axon where it can elicit action potentials. The driver potential can integrate with synaptic input to structure the final impulse burst (Benson and Cooke, 1984). In the untreated, intact neurone this

event can be recorded in the soma as a plateau potential. A schematic representation of driver potential production and the events leading to the generation of plateau potentials in cardiac neurones is given in Fig. 1.4.

It must be stressed that, as with molluscan bursting neurones, a uniform ionic mechanism is unlikely to account for bursting activity recorded from all types of crustacean neurones. Differences between individual neurone-types are likely to exist, both in terms of the topographical organization of burst-forming membrane, and in terms of the ionic basis for plateau or driver potentials. There is evidence pointing to differences in the net inward current carrying the driver potentials of 'large' and 'small' cells of the cardiac ganglion of *Homarus*: these two cell-populations appear to respond differently to changes in external divalent cations and, whilst driver potentials in the large motoneurones appear to be carried largely by calcium ions, some of the inward current carrying these events in the smaller pacemaker cells appears to utilise an ion other than calcium (Berlind, 1987). It also appears that characteristically different bursting patterns induced by different amines in the AB cell of the lobster STG may be accounted for by different burst generating mechanisms in the same neurone (Harris-Warrick and Flamm, 1987).

FIGURE 1.4: Bursting in crustacean cardiac ganglion neurones. (A) Spontaneous activity (upper trace) and an evoked driver potential (middle trace) recorded from Cell 1 of *Homarus americanus*. The driver potential was evoked by a brief depolarising pulse (lower trace), following perfusion of the preparation with 3×10^{-7}M tetrodotoxin (TTX). (B) Diagram representing the integrative region of the soma and proximal axon where depolarising influences (such as pacemaker depolarisations, electrotonic and chemical synaptic inputs) can evoke driver potential production and integrate with it to drive a burst of impulses at the more distal spike initiating zone.

This figure does not contain original data. (A) and (B) are from Benson and Cooke (1984).


A
Normal
Impulse
1
EPSP
−45 mV
−55 mV
TTX
Driver potential
300 ms
B
Σ { Stretch depol.
Pacemaker depol.(?)
+ Driver potential +
Electrotonic depol.
+ EPSPs (×4)
Trigger Zone
Impulses

1.4.3. Vertebrate Bursting Neurones

Neurones from vertebrates, including mammals, can exhibit bistability (see section 1.2 for examples). An obvious question is whether the ionic mechanisms underlying bistability in vertebrates relate to those which occur in crustacea or molluscs. This is a difficult issue to address comprehensively, since no vertebrate preparation has appeared singularly appropriate for this study in quite the same way as, for example, R_{15} from *Aplysia* or crustacean cardiac motoneurones. As a consequence, a vast amount of information exists, but has been gathered from a number of different sources.

Adams and Benson (1989) use selected examples to draw parallels between vertebrate bursting neurones and those from crustacea and molluscs. It appears that in many respects the ionic mechanisms are similar; this is not particularly surprising, in view of the fact that different cell types exhibiting bistable behaviour share similar properties evident in their current-voltage relationship (see section 1.3).

One common property is that an inward calcium current appears to be involved in many (although not all) bistable events from vertebrates. For example, plateau potentials in turtle motoneurones have been shown to be mediated by a non-inactivating calcium conductance, sensitive to the calcium channel blocker nifedipine (Hounsgaard and Kiehn, 1989) and it has

been proposed that a similar mechanism underlies plateaux in α-motoneurones from the cat (Hounsgaard *et al*, 1988). Similarly, plateau potentials from hypothalamic neurones also appear calcium-dependent (Legendre *et al*, 1982). Plateaux from hypothalamic neurones exhibit a slow repolarisation during the plateau phase; this appears to involve potassium conductances; but the rapid repolarisation which terminates the event may be a result of inactivation of an inward current (Legendre *et al*, 1988) However, in contrast to some molluscan cells, potassium currents probably do play a role in repolarising the neurone sufficiently to permit inactivation of the calcium current. The refractory period following plateaux in these neurones appears to depend on calcium activated potassium conductances, although there is no afterhyperpolarisation (Legendre *et al*, 1988).

Calcium entry and activation of calcium-dependent potassium currents also appear to be important in generating oscillatory properties of inferior olive neurones. In these neurones, an additional calcium conductance which is inactive at the normal resting potential is deinactivated by the I_C-induced hyperpolarisation, and as the membrane depolarises towards its original membrane potential a low threshold calcium spike is generated (Llinás and Yarom, 1981a,b; review: Llinás, 1989). Similar, low threshold (T-type) calcium currents may play an

important role in the formation of bursting behaviour in a number of mammalian neurones: thalamic neurones undergo a shift from tonic firing to patterned bursting upon hyperpolarisation which appears to be mediated by a T-type current (Suzuki and Rogawski, 1989) and indirect evidence implicates changes in T-current to the development with age of bursting behaviour in dorsal root ganglion neurones (White *et al*, 1989; Lovinger and White, 1989).

1.5. Modulation of bursting activity and its implications

Rhythmic oscillations from spontaneously bursting neurones, conditional oscillators and neurones demonstrating plateau properties are subject to modulation (see reviews by Benson and Adams, 1987; Dickinson, 1989; Katz and Harris-Warrick, 1990).. This has important implications for the output of a wide range of neural networks.

The bulk of information on modulation of intrinsic bursting activity comes from work investigating actions of neuromodulators and neurotransmitters on bistable, or oscillatory properties.

The pattern of activity of spontaneously bursting neurones such as *Aplysia* R_{15} can be enhanced or suppressed by various neuroactive peptides (among other substances). Serotonin and dopamine both suppress bursting in R_{15}, but do so by different mechanisms (see Benson and Adams, 1987 for a review). Serotonin appears to increase the anomalous rectifying potassium conductance (I_{Kr}). This conductance permits inward ion movement at highly negative potentials, but is an imperfect rectifier. This means that it passes a small outward current at potentials up to 30mV positive to E_K (Levitan and Kaczmarek, 1987). By increasing I_{Kr} (and thereby the magnitude of this small outward current), serotonin can actually shift

the I-V relationship of R_{15} closer to the 'zero-current' axis (making the interburst hyperpolarisation greater). If the serotonin concentration is large enough, the I-V curve intersects the 'zero-current' axis (with positive slope) and the neurone is rendered quiescent. Dopamine, on the other hand, reduces the negative slope region of the I-V curve by decreasing I_{NSR}. As a result, the I-V curve crosses the 'zero-current' axis with positive slope at potentials negative to the threshold for action potential production, rendering the neurone stable and quiescent.

In contrast, many neurones cannot generate bursting activity or plateau potentials in the absence of some tonic, unpatterned modulatory input; in such cases rhythmic impulse generation is a 'conditional' property. Perhaps this is where the modulation of intrinsic rhythmic capability is most interesting. For example, when modulatory inputs from other ganglia are removed, the anterior burster (AB) neurone of the lobster loses the ability to act as the pacemaker neurone for the pyloric rhythm of the STG, but when these inputs are stimulated the AB neurone demonstrates bursting activity (see Harris-Warrick and Flamm, 1987). When isolated from synaptic input the AB neurone is quiescent, but it responds to the amines dopamine, serotonin and octopamine by generating rhythmic bursting activity. Each amine generates a unique activity pattern since they each activate

different mechanisms of burst-generation: bursting induced by serotonin or octopamine is dependent on sodium influx through TTX-sensitive channels, whilst dopamine-induced bursting is TTX-insensitive but is more sensitive to alterations in external calcium concentration (Harris-Warrick and Flamm, 1987).

Proctolin can also induce bursting in the quiescent AB neurone and increase the amplitude and frequency of the oscillation in active neurones. These effects can outlast the presence of proctolin for minutes or hours (Hooper and Marder, 1987). Such long-lasting effects suggest that these actions of proctolin may involve some kind of second messenger system.

Tonic potassium conductances may provide additional targets for activity modulation in this neurone since bursting can be induced in the quiescent neurone by various potassium channel blockers (Harris-Warrick and Johnson, 1987).

Such multiple mechanisms of burst-induction in the pacemaker neurone for the pyloric rhythm provide the means for diverse ways in which this activity can be initiated in the intact animal. Moreover, an interplay between different modulators could induce subtle changes in phasing of the rhythm; the effects of any given neuromodulator will depend on previous alterations in activity by other modulators (Dickinson, 1989).

Work on the STG system has revealed a further, dramatic role for the modulation of intrinsic rhythmic properties: modulation may serve to reconfigure neurones within a neural network, or switch individual neurones between circuits generating different behaviours. Evidence for the former role has been obtained for the lobster STG, where stimulation of the APM (*in vitro*) can change one pattern of activity (the 'cut and grind' pattern) into another (the 'squeeze' pattern), in part by altering plateau-properties of constituent neurones (Nagy *et al*, 1988; Dickinson *et al*, 1988). In contrast, injection of proctolin into the haemolymph of the animal results in induction of the 'cut and grind' pattern (Heinzel, 1988a,b). There is also evidence that one pyloric neurone can be removed from the circuit generating the pyloric rhythm by stimulation of a sensory nerve (this results in a temporary loss of its oscillatory properties) and can be recruited into the separate cardiac sac rhythm (Hooper and Moulins, 1989).

The ability of neurones to express plateau potentials appears to be 'conditional' in many neurones from both invertebrates and vertebrates. In the first studies of plateau activity performed on the STG (Russell and Hartline, 1978), plateau potentials from some neurones were enhanced following stimulation of the input (stomatogastric) nerve, while in others (pyloric

constrictors) plateau properties vanished in the absence of central inputs .

Dickinson and Nagy subsequently determined that stimulation of the anterior pyloric modulator (APM) unveils plateau properties in the pyloric constrictors and that this modulation alters the response of the pyloric constrictors to synaptic input (Nagy and Dickinson, 1983; Dickinson and Nagy, 1983). The modulatory effect of the APM was observed to be mediated through cholinergic receptors. Plateau potentials in the pyloric constrictor neurones are able to drive action potentials; therefore the ability of the pyloric constrictor neurones to generate plateau potentials can contribute to the shaping of the pyloric motor pattern. The APM is currently one of five identified neurones which are known to provide modulatory input to the STG and, interestingly, whilst it can enhance plateau properties of some STG neurones it suppresses plateaux in at least one (for a review see Katz and Harris-Warrick, 1990).

The plateau properties of some vertebrate neurones also appear to be expressed only under certain circumstances. For example, it has been shown that fictive locomotor activity in the lamprey is associated with NMDA-induced oscillations in cells active during this behaviour (Wallén and Grillner, 1987). These oscillations are an intrinsic property of the neurones examined (they persist in TTX which is presumed to abolish synaptic input), but are not

apparent unless an NMDA receptor agonist is present. Similarly, the ability of α-motoneurones from the cat to generate plateaux depends on non-patterned input from descending serotonergic and noradrenergic fibres (Hounsgaard *et al*, 1988; Crone *et al*, 1988; Conway *et al*, 1988). Motoneurones located below an acute spinal transection are unable to generate plateaux in the absence of intravenously administered serotonergic and noradrenergic precursors. These findings correspond well with observations made using the isolated spinal cord of the turtle, which show that serotonin induces plateau properties in motoneurones (Hounsgaard and Kiehn, 1989). In the cat the descending fibres may be considered as exerting a permissive effect on α-motoneurone properties, much in the same way as the APM neurone acts upon pyloric constrictors of the crustacean STG system. Descending input to the motoneurones allows them to respond to a brief excitatory input with a relatively long-duration burst of action potentials, and therefore induces a powerful synaptic amplification system in these cells.

In summary, the modulation of intrinsic oscillatory and bistable properties by non-patterned synaptic or humoural influences almost certainly has key importance to the generation of many rhythmic behaviours. Such modulation can affect the phasing and timing of rhythmic impulse generation in central pattern generating networks. It can reconfigure

neurones within a network or, more spectacularly, switch a neurone between neural networks. It can also alter the sensitivity of locomotor motoneurones (which are not part of the CPG) to synaptic inputs, providing an important synaptic amplification system. Thus, the modulation of intrinsic oscillatory/bistable properties may produce plasticity within neural networks which generate rhythmic behaviours.

1.6. Insects and the study of locomotor pattern generation

Insects exhibit two forms of locomotion (flight and walking) which have long been of interest to behavioural neuroscientists. In fact, electrophysiological studies of insect flight carried out in the early '60s form an important land-mark in our understanding of pattern generation. Wilson (1961) demonstrated that the basic pattern of motor output during locust flight could be generated from a central nervous system which is isolated from sensory feedback (see Wilson, 1965), and this became a powerful piece of evidence in the case for rhythmic behaviour being centrally generated. A similar situation seems to prevail during cockroach walking: Pearson and Iles (1970) were able to record rhythmic bursts of activity from stumps of leg muscles in the absence of any sensory input (for reviews see Delcomyn, 1980; 1985).

However, despite considerable interest in both insect flight and walking, detailed intracellular studies initially proved difficult. It was difficult to identify the cell bodies of individual neurones in insect preparations visually (unlike many molluscan preparations) and this situation was exacerbated by the lack of intracellular dyes. Thus, whilst certain large insect axons lent themselves to the study of membrane properties (see Narahashi, 1965 and Pitman,

1985 for reviews), it was difficult to make intracellular recordings from identified neurone somata. Intracellular recordings were obtained from groups or clusters of neurones (Hagiwara and Watanabe, 1956; Callec and Boistel, 1966, 1967; Kerkut *et al*, 1968; Rowe, 1969) but individual neurones remained unidentified. This, in turn, meant that it was difficult to construct neural maps and to assess the role of individual cells in the generation of specific behaviours (a situation somewhat lamented by Hoyle, 1970).

In 1970, Hoyle and Burrows were able to locate motoneurones supplying the metathoracic leg of the locust, *in vivo*. Their experiments involved a combination of direct stimulation and intracellular injection of the dye Procion yellow (see Hoyle, 1970). Subsequently the morphology and physiology of these neurones was investigated in more detail (Hoyle and Burrows, 1973; Burrows and Hoyle, 1973). At about the same time (1971) Iles and Mulloney found that the dye Procion Yellow could be introduced into the cut end of axons, allowing staining of even small neurones; and in 1972 Pitman Tweedle and Cohen demonstrated that neurones could be stained, and subsequently observed in whole-mount preparations, by the introduction of cobalt ions and precipitation of cobalt sulphide (Pitman *et al*, 1972a). Thus, it became possible to identify individual neurones in ways that had not been previously possible.

Many of the neurones which are active during insect locomotion (particularly flight) have now been identified (reviewed by Robertson, 1986). However, despite the considerable attention locomotor activity has received, both in the intact and deafferented nervous system (for example: Wolf and Pearson, 1989; Robertson and Reye, 1988; and Robertson 1990), no endogenously bursty cells have been found and rhythmic activity appears to result from the arrangement of excitatory and inhibitory connections in the neural networks responsible for these behaviours. Moreover, motoneurones themselves are reported as 'simple output elements' which do not play a role in timing or phasing of the motor pattern; therefore it seems that the locomotor rhythm is centrally generated (Robertson, 1986). This, of course, contrasts quite markedly with other systems where there is considerable evidence that spontaneous and conditional bursting neurones act as pacemakers for rhythmic pattern generation, and where bistable membrane properties may allow motoneurones which are not part of the central pattern generator to shape the centrally generated rhythm.

Before proceeding to describe the results obtained in the present study two further brief background sections are necessary. The first section discusses,

in fairly general terms, the organization of the insect nervous system and of neurones within the neural ganglia. The second section discusses membrane properties of insect motoneurones, since this type of neurone forms the focus for the work.

1.7. The insect nervous system

The insect central nervous system (CNS) consists of segmentally arranged ganglia, joined together by paired interganglionic connectives (Fig. 1.5A). The largest of these is the supraoesophageal ganglion, or brain, which receives inputs from the compound eyes, ocelli, antennae and mouthparts. The first ganglion of the ventral chain is the suboesophageal ganglion; this receives sensory inputs from mouthparts and cuticle of the head and neck, with outputs to the salivary glands and muscles of the head and neck. Three thoracic ganglia innervate the sensilla and muscles of the thorax. These also serve the three pairs of legs; each leg-pair is located at the level of the appropriate ganglion (one leg on either side of the ganglion). Caudal to the thorax, a number of smaller abdominal ganglia provide sensory and motor innervation to viscera, genitalia, skeletal muscle and cuticle of the abdomen. The paired interganglionic connectives contain axons, although no synaptic contacts are made within the connectives. Some of

these axons run along the entire length of the nerve cord uninterrupted. Others, in contrast, merely extend between adjacent ganglia.

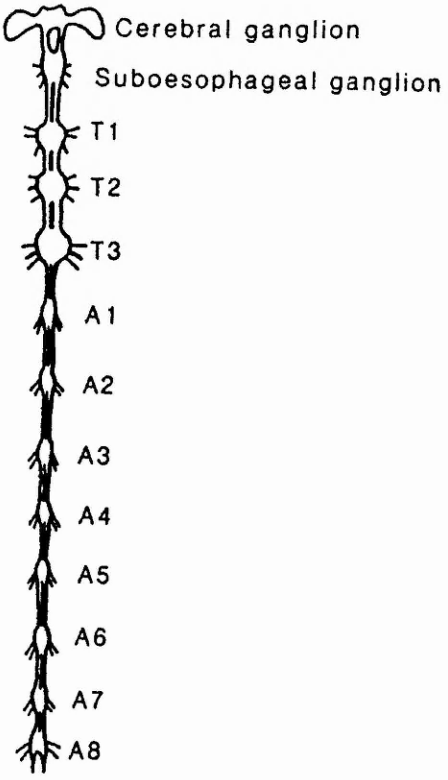
The central nervous system is surrounded by a nerve sheath. This is divided into an outer acellular neural lamella, comprised of collagen fibres in a polysaccharide matrix, and an inner perineurium: a single layer of specialized glial cells. This sheath, which can be likened the mammalian "blood brain barrier", may exhibit selectivity. It appears to restrict the exchange of materials between the haemolymph and the CNS , particularly large, polar, or charged molecules (Treherne and Pichon, 1972).

Each ganglion has a complex central neuropile. This area is highly organized, and it is here that synaptic contacts between dendritic and axonal branches are made. The neuropile is surrounded by a "cortex" of neuronal cell bodies (somata) and glial cells (Fig. 1.5B). Although the somata of motoneurones and interneurones lie within the CNS, those of most sensory neurones lie in the periphery. There are no synapses on the neuronal cell bodies (Bullock and Horridge, 1965).

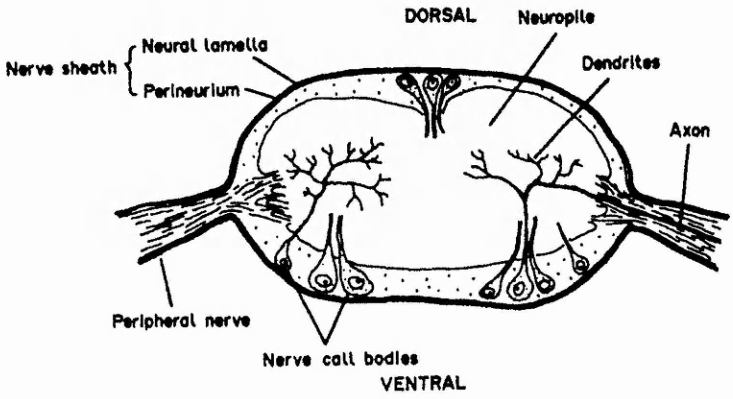
FIGURE 1.5: (A) Schematic drawing of an insect nerve cord (from Gifford, 1989). T1-3: thoracic ganglia. A1-8: abdominal ganglia.

(B) A transverse section through a typical ganglion of the insect ventral nerve cord (from Pitman, 1985) showing the location of neurone somata relative to the ganglion surface. The neuropile contains the processes from hundreds or thousands of neurones. The axon of one neurone is shown leaving the ganglion *via* a peripheral nerve.

A


Cerebral ganglion
Suboesophageal ganglion
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8

B


DORSAL
Neuropile
Nerve sheath
Neural lamella
Perineurium
Dendrites
Axon
Peripheral nerve
Nerve cell bodies
VENTRAL

1.8. Membrane properties of insect motoneurones

Axons of insect motoneurones, sensory neurones and many interneurones are excitable; they possess fast sodium channels and generate sodium-dependent action potentials (Narahashi, 1965; for a review of pharmacological properties see Pelhate and Sattelle, 1982). This is not the case for all neurones, however, since some interneurones appear to lack the ability to generate action potentials altogether (Pearson and Fourtner, 1975). Such interneurones can exert powerful effects on post-synaptic neurones *via* synaptically driven oscillations in their membrane potential and appear to play important roles in central generation of locomotor rhythms (Pearson and Fourtner, 1975; Robertson, 1986).

Insect neurones can possess somata which exhibit membrane properties differing considerably from those of their axons. Some neurones possess excitable somata, many do not. The somata of a group of cells located along the dorsal midline of cockroach and locust ganglia (dorsal unpaired median (DUM) neurones) can generate overshooting action potentials (Callec and Boistel, 1966, 1967; Kerkut *et al*, 1968; Jėgo *et al*, 1970) which appear to be carried largely by sodium ions (Jėgo *et al*, 1970), but may be partly calcium-dependent (Goodman and Heitler, 1979). In contrast, the somata of motoneurones seem to be inexcitable; often only relatively small, attenuated action

potentials can be recorded from cell bodies, which indicates that action potentials are generated in electrically distant parts of the neurone and that these are not actively propagated towards the cell body (Pitman, Tweedle and Cohen, 1972b; Hoyle and Burrows, 1973, Gwilliam and Burrows, 1980).

Motoneurones can, however, exhibit delayed rectification and many cockroach motoneurones can generate an active response: depolarising current injection can produce a series of damped membrane oscillations which are graded, increasing with the magnitude of applied depolarisation (Pitman, 1979). Under voltage-clamp, several outward conductances are apparent. A significant proportion of the outward current seen during depolarisation is activated by calcium influx. The current-voltage (I-V) relationship of some cells demonstrates an 'N'-shape at positive voltage which is sensitive to calcium-free saline and calcium and potassium channel blockers; this component of the outward current is attributable to a calcium-mediated potassium conductance, I_c (Thomas, 1984; Nightingale and Pitman, 1989). A portion of the rectifying current is independent of calcium movement; this persists in the presence of calcium channel blockers and calcium-free saline, but is greatly reduced by external application of TEA (Nightingale and Pitman, 1989; Thomas, 1984). A third, transient outward current component can only be seen after a hyperpolarising pre-pulse and, therefore,

is partially or completely inactivated in the range of normal resting potentials (Nightingale and Pitman, 1989). This component may correspond to the transient outward current (I_A) described for molluscan neurones by Connor and Stevens (1971).

The presence of a number of strong outward currents in the cell bodies of insect motoneurones seems to mask the effects of any inward currents originating from the cell body. A number of experimental observations strongly support the proposition that Ca-channels are normally present in cell bodies. Motoneurones can generate a calcium-dependent outward current (Thomas, 1984; Nightingale and Pitman, 1989); since this is sensitive to transmembrane calcium movement, the rise in intracellular calcium which drives potassium efflux requires calcium channels in the cell membrane. Further evidence for this comes from the direct observation of voltage-sensitive calcium channels, using patch-clamp, in unidentified cockroach neurones (Christensen *et al*, 1988). Furthermore, under certain circumstances motoneurone somata can support all-or-none calcium-dependent action potentials. External TEA application and intracellular citrate injection allow the cockroach 'fast' coxal depressor motoneurone to respond to depolarisation with calcium-dependent action potentials, an action attributed to a reduction in suppressive outward currents (Pitman, 1979). TEA application has similar effects upon the 'fast'

extensor tibiae neurone of the locust, although part of the current carrying the action potential may be carried by sodium ions (Goodman and Heitler, 1979). Thus, whilst channels which can mediate fast, calcium-dependent events are normally present in these cell bodies, in the absence of pharmacological intervention these channels cannot carry sufficient depolarising current to overcome the more powerful and rapidly developing outward currents.

The excitability of insect motoneurones can also be enhanced by other treatments. Sodium-dependent action potentials can be recorded from the soma of the 'fast' coxal depressor motoneurone, with a delay of several days, following axotomy or pretreatment of the animal with colchicine (Pitman, Tweedle and Cohen, 1972b; Pitman, 1975) or, with a shorter delay, following a period of anoxia (Pitman, 1988). Axotomy and colchicine treatment have similar effects upon the soma of the locust 'fast' extensor tibiae neurone (Goodman and Heitler, 1979). The delay between these treatments and the onset of their effects indicates that the sodium channels which carry the action potentials are normally absent from the cell body, but can be incorporated into soma membrane. This could be explained in different ways. Firstly, these treatments may increase the *de novo* synthesis of sodium channels. These extra sodium channels are surplus to requirements and are incorporated into the cell body (Goodman and Heitler, 1979). Such a

mechanism could be particularly important in the hyperexcitability observed following axotomy; if the axon has been severed it cannot incorporate sodium channels, resulting in an increase in somatic channel density (Wiens and Atwood, 1982). There is evidence linking the development of hyperexcitability with alterations in protein synthesis: perinuclear RNA increases significantly in cockroach neurones following axotomy and colchicine treatment (Pitman *et al*, 1972b). Secondly, hyperexcitability could result from the incorporation of previously-synthesized channels into the cell membrane from the cytosol, or a long-term change in some intracellular regulatory process (such as pH regulation). These alternatives to increased channel synthesis may be involved in producing the delayed effects of anoxia; for there appears to be no significant increase in protein synthesis in neurones from post-anoxic animals relative to controls (Pitman, 1988) At present the precise ways in which these different treatments exert their effects are unknown.

In summary, it appears that the cell bodies of insect motoneurones are not normally excitable, although excitability can be enhanced by a variety of treatments which act in different ways. Some procedures reveal channels which are normally present, while others may lead to the expression of new channel types in the soma membrane.

1.9. Aims of this thesis

The aims of this thesis were:

i) To re-examine the membrane properties of an identified motoneurone from the cockroach (*Periplaneta americana*), focusing particularly upon identifying any non-linear properties which may be present in the untreated neurone.

ii) To use two-electrode current- and voltage-clamp recording techniques to characterise any non-linear events found in this neurone.

iii) To extend the search to other identified neurones from the cockroach and locust (*Schistocerca gregaria*).

CHAPTER 2. MATERIALS AND METHODS

2.1. Animals: maintenance and dissection

Most experiments were performed on cockroaches drawn from a laboratory culture of *Periplaneta americana*. A breeding colony was maintained at 27°C in plastic containers and fed variously on 'Minced Morsels' dog food and bran-meal whilst being constantly supplied with water. When necessary this colony was supplemented with animals obtained from a commercial supplier (Blades Biological (Kent)). All experiments were performed on adult males, in part to ensure the viability of the breeding colony, but foremost to avoid any effects of sexual dimorphism. In a small number of experiments locusts were used; these were drawn from a laboratory culture of *Schistocerca gregaria* maintained at 27°C. Locusts were fed on barley-seedlings and bran-meal and were supplied with water.

Animals were sacrificed by decapitation and, for most experiments, their legs removed prior to dissection (thereby making access to the ventral mid-line of the animal somewhat easier). They were pinned, ventral surface uppermost, on a Sylgard dish. The ventral nerve-cord, which lies just beneath the cuticle, was then exposed and dissected out into normal cockroach (or locust) saline (APPENDIX I). For experiments utilising

extracellular recording techniques the pro- and mesothoracic leg-pairs were removed as in the normal dissection, but the metathoracic legs severed distal to the coxae. Dissection then proceeded as normal, with the exception that the metathoracic Nerve 5 was followed out from the metathoracic ganglion to the coxae and dissected away from the animal with the ventral nerve cord. Dissections were performed under a Kyowa zoom binocular microscope (70X - 45X).

For electrophysiological work the nerve cord was placed on a small, transparent, perspex slide (10mm x 25mm) with the ventral side uppermost on an Araldite ridge (Fig. 2.1). The nerve cord was anchored to this slide with small plastic bands. One or two drops of methylene blue (approximately 4%) were dropped onto the surface of the ganglion with a fine-gauge syringe needle in order to visualise the protective neural sheath. This was then mechanically removed and the preparation immediately transferred to a perspex experimental chamber.

For experiments utilising surgically isolated neurone somata, the cell body of the cockroach fast coxal depressor motoneurone, D_f (Pearson and Iles, 1970), was mechanically isolated *in situ*. This procedure (described below) was performed once the metathoracic ganglion had been desheathed and the preparation transferred to the experimental chamber. The area

surrounding D_f was washed with a fine jet of saline from a broken microelectrode; this cleared the region of surrounding glial cells and partially freed the cell body. A sharpened tungsten needle, or entomological pin was used to cut beneath the neurone soma, severing the initial process of the neurone and leaving the cell body isolated from the axon and dendritic processes. The effectiveness of this procedure was routinely tested by filling the neurone with the flourescent dye Lucifer yellow; in undercut cells flourescence was restricted to the soma, whilst unsuccessfully undercut cells also demonstrated flourescence in their axons. Data from cells where the isolation procedure proved unsuccessful were discarded.

For a small number of experiments, animals were exposed to a period of anoxia prior to electrophysiological recording. Animals were placed in a sealed container with a moistened cotton-wool ball, then gassed with pure carbon dioxide. Animals were exposed to CO_2 for between one and two hours, after which time they were allowed to recover in individual boxes supplied with water and food. Between 24 and 48 hours after exposure to CO_2, animals were removed and prepared for investigation as described above.

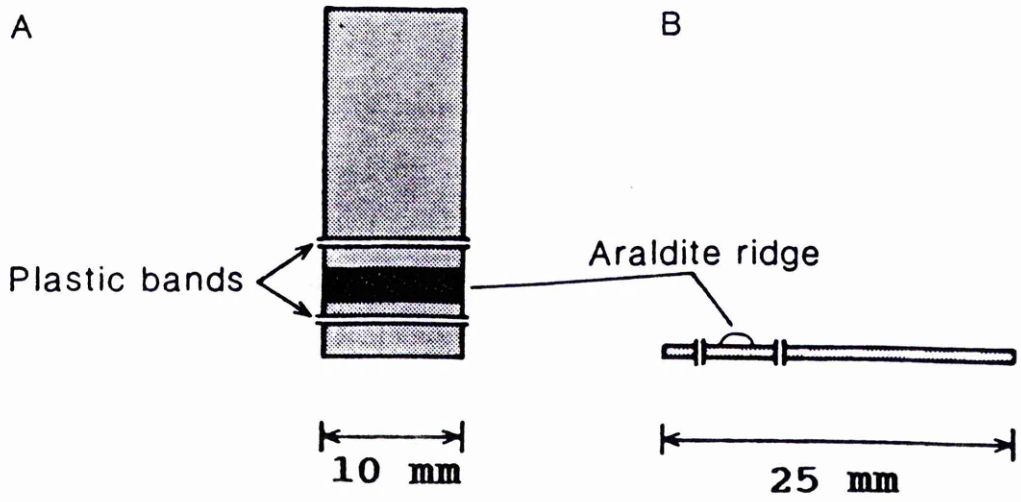


FIGURE 2.1: The preparation slide. (A) Front view. (B) Side view. The nerve cord was positioned on this slide in such a way that the metathoracic ganglion lay on the Araldite ridge. The nerve cord was secured on either side of this ridge by plastic bands. (Figure altered from: Nightingale (1988)).

2.2. Electrophysiology

2.2.1. Experimental chamber

The experimental chamber (Fig. 2.2) was constructed from a rectangular perspex block (81 X 25 X 36 mm). The chamber was perfused with oxygenated, circulating saline; pure oxygen (BOC) was supplied through a narrow plastic tube placed in the right hand compartment of the chamber. An anticlockwise circulation of saline was established by the rising oxygen bubbles. The level of saline was kept constant by surface suction applied to the left hand compartment, ensuring a constant bath volume of 2ml. The experimental slide was placed in the central compartment of this chamber; pharmacological agents, saline and oxygen were added to a separate, connected compartment, thereby limiting addition artefacts and movement of the preparation.

The virtual earth electrodes were connected with the experimental chamber via an Agar bridge. This arrangement effectively isolated the environment of the bath electrodes from ionic changes to the superfusate in the main chamber. Bath electrodes were made from silver wire (1mm diameter) which was hammered flat (2-3mm wide) to increase the conducting surface area. These electrodes were electrolytically coated with chloride, using 1-2M potassium chloride and a 9V battery (bath electrode as anode). This procedure prevented

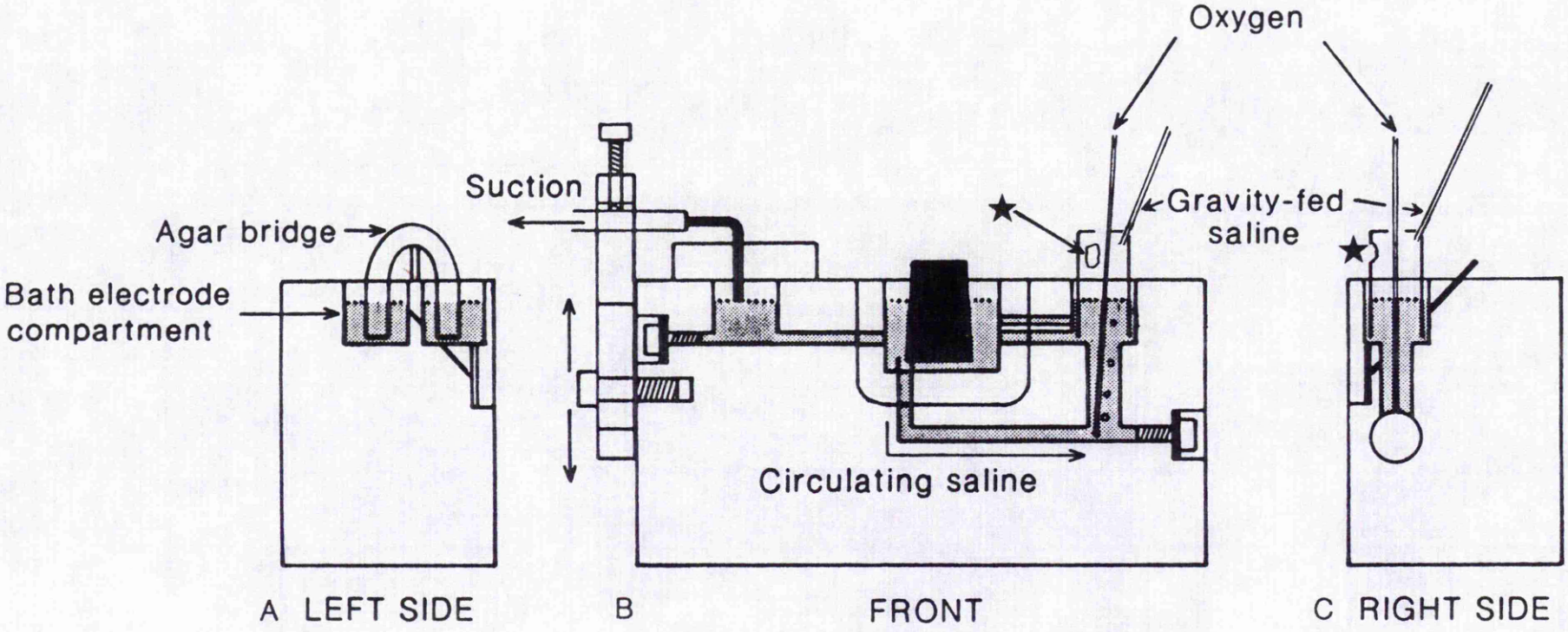
electrical polarisation of the electrodes, thereby allowing the recording of stable potentials.

All drugs were directly applied to the right hand compartment of the experimental chamber. All drug concentrations referred to in the text are final bath concentrations. Drugs were dissolved in normal saline. Drugs were supplied by Sigma Chemical Co. (Ltd.).

2.2.2. Illumination

The preparation was illuminated using a beam from a laboratory-built lamp (the light source was a 12V, 55W halogen bulb) located outside the Faraday cage, which reflected off a mirror inside the cage. The reflected beam was focused down onto the preparation by a convergent lens which could be moved in one plane. A binocular zoom microscope (Nikon) could be swung into position in front of the bath, once dissection was complete, and the illuminated preparation viewed at magnifications between 18X to 80X.

FIGURE 2.2: The experimental chamber showing (A) left, (B) front and (C) right side views. Areas containing saline are shaded with 'stippling'. Saline enters the chamber under gravity; an anti-clockwise circulation is established as a result of oxygen bubbling in the right hand compartment. The direction of circulation is indicated by an arrow. The saline is maintained at a constant level a by a surface suction apparatus. The asterisks in (B) and (C) mark the site of addition of pharmacological agents. (Figure modified from: Nightingale (1988)).


Oxygen
Suction
Agar bridge
Gravity-fed saline
Bath electrode compartment
Circulating saline
A LEFT SIDE
B
FRONT
C RIGHT SIDE

2.2.3. Neurone identification

In some molluscan preparations different neurone types are easily identified on the basis of colour, as well as topographical location and electrical behaviour (for example: Slade *et al*, 1981). Insect preparations are not afforded this feature. The desheathed metathoracic ganglion (T3) of the isolated nerve-cord is of near-uniform colour, and so visual identification of neurone somata, prior to electrophysiological recording, must be on the basis of their size and location relative to landmarks that are relatively consistent from one animal to the next.

The cockroach D_f is readily identified. It possesses one of the largest cell bodies on the ventral surface of the metathoracic ganglion which, for each of the heterolateral pair, lies in approximately the same location relative to large, Y-shaped tracheal processes which run close to the surface of the ganglion. The soma of the neurone can be visualised using incident light and can be distinguished from the smaller cell bodies of its neighbours. In carefully desheathed preparations, the somata of cells 27, and 28 (D_f) lie juxtaposed, cell 27 is smaller and normally lies medial to D_f (for complete map see Cohen and Jacklet, 1967). Fig. 2.3 shows a toluidine-blue stain of the ventral surface of T3. (see APPENDIX II for experimental protocol). Cell bodies are coloured violet. Initially the cell bodies of both neurones and glia take up the

dye; however, the differentiation process clears smaller cells (such as glia) before larger cells (larger glia remain stained). D_f is labelled with an arrow and is clearly the largest cell stained. Cell 27 is less well defined, but is also marked.

FIGURE 2.3: Photograph showing toluidine-blue staining of neurones on the ventral surface of the third thoracic ganglion (T3). Cell bodies are coloured violet. The large anterior connectives can be seen leaving T3 (top of picture); the posterior connectives are also shown leaving T3 and join with the first abdominal ganglion, part of which is visible at the base of the picture. The soma of the left D_f (cell 28) is marked with an arrow. The soma of the left cell 27 is less clear; it is medial to that of D_f and is marked with an arrow-head. The relatively large unstained areas contain neuropile. Horizontal scale bar is 0.5mm.

2.2.4. Intracellular recording

A pair of Prior micromanipulators fitted with reduction drives in two orthogonal horizontal planes were used to position microelectrodes in the bath. Microelectrodes were drawn from 1mm internal diameter thin walled filament glass (Clark Electromedical Instruments) using a Narishige PE-2 vertical puller, inclined to approximately 45°. These electrodes were filled with 2M potassium acetate. For current-clamp and voltage-clamp recordings both recording and current-passing electrodes had resistances of 14-20 MΩ.

The recording-microelectrode holder was attached to the amplifier headstage via a screened cable; the screen was connected to a central earth point on the base-plate. The current-electrode was connected to a laboratory made switching mechanism which allowed the microelectrode to be connected (1) to the headstage of a second amplifier, (2) directly to a D.C. powered stimulator (Digitimer DS2), or (3) to the output stage of the voltage-clamp amplifier.

For penetration of neurone somata, the illumination was altered by adjusting the position or angle of the focussing-lens to highlight the profile of the cell body. The microelectrode tips were then lowered into the saline and positioned over the cell surface. Before proceeding with impalement, the electrode resistances were checked and the electrodes balanced through the

amplifier bridge circuits. 0mV was then set by adjusting the amplified voltage deflection on the oscilloscope (Gould 1604) so that its position was equivalent to that of the beam when the oscilloscope input was taken to 'ground'; this allowed recordings of membrane potential to be taken relative to a predetermined zero-point. For paired microelectrode experiments the recording electrode was first lowered until a depression in the cell surface was evident; this corresponded to a small voltage deflection. A series of brief (approximately 30ms) hyperpolarising pulses (between -80nA and -100nA) were applied in order to facilitate impalement which was indicated by a sudden negative shift in membrane potential of between -60mV and -70mV. The second electrode was lowered onto the cell whilst regular 50ms, -100nA pulses were passed into it. The membrane potential was monitored using the recording electrode and penetration was marked by large hyperpolarising responses corresponding to the pulses applied through the second microelectrode. Once both microelectrodes were in place, an earthed brass shield was introduced between them and lowered as near to the surface of the saline as possible, using a third micromanipulator (Narishige), in order to reduce inter-electrode capacitative coupling.

2.2.5. Extracellular recording

In some experiments a suction electrode was used to take recordings from the fifth nerve (N5) of the metathoracic ganglion in order to record impulses from the axon of D_f (and in one series from the axon of the locust 'fast' extensor tibiae motoneurone (Hoyle and Burrows, 1973)). A suction electrode was held in the Narishige micromanipulator (this usually held the earthed brass shield) behind the preparation bath, and could be lowered into the saline without restricting movement of either microelectrode. The tip of the suction electrode was positioned over the severed end of N5 and gentle back pressure applied using a 5ml syringe attached to the barrel of the electrode, thereby drawing N5 up into the barrel. To record from D_f it was important to ensure that a sufficient length of nerve was drawn into the suction electrode so that the branching point N5r1 (Pearson and Iles, 1970) was within the electrode barrel, since it is this branch-point that contains the axon of D_f.

For recordings from N5, the suction electrode barrel and return-path were connected to the two terminals of the input stage of a fixed gain (1000x) extracellular amplifier (Isleworth A103).

For some experiments a suction electrode was used to identify the locust fast extensor tibiae motoneurone by antidromic stimulation of its axon. To stimulate <u>via</u>

the suction electrode, the barrel of the electrode was connected to the positive terminal of a stimulator (S44, Grass Instruments) and the return path connected to the negative terminal.

(Block diagrams of intracellular and extracellular instrumentation are given in APPENDIX III)

2.2.6. Current-clamp

Under current-clamp, amplified extracellular or intracellular recordings were displayed on a Gould 1604 4-channel digital oscilloscope. During experiments current pulses were applied to cells *via* the second microelectrode using a DC powered stimulator (Digitimer DS2), triggered by a laboratory built pulse generator. Applied current was measured in terms of current flowing to earth through the preparation bath, using a virtual earth current monitor of switched gain; the output of this device was displayed on a third oscilloscope channel.

In some current-clamp experiments citrate ions were ionophoretically injected into the soma of D_f. In these experiments the voltage recording electrode was filled with 2M potassium acetate; the current electrode, however, was filled with 2M potassium citrate. Citrate ions were injected into the neurone soma by passing regular (1 Hz), 500ms duration hyperpolarising current

pulses of sufficient magnitude to hyperpolarise the neurone by 30mV. Citrate ions were injected into neurones for 5-15 minutes.

2.2.7. Voltage-clamp

For voltage-clamp recordings, the output of the intracellular-recording amplifier was fed directly into a laboratory-built voltage-clamp amplifier (a simplified circuit diagram is given in APPENDIX III). The compliance of this amplifier was ± 120V in order to achieve fast voltage-clamps. The output of the voltage-clamp amplifier could be passed into the current microelectrode *via* a switch, which allowed one to easily opt between current- and voltage-clamp.

Command-pulse regimens were generated either manually (APPENDIX III), or were computer driven. Command signals were fed into the command-input of the voltage-clamp amplifier. For manually driven experiments, the output of the stimulus-isolation-unit of a mains powered stimulator (S44, Grass Instruments) was passed into a manually operated step-attenuator and from there to the command input of the voltage-clamp amplifier. For some computer-controlled experiments, command signals were controlled by a UNILAB interface (532.001) and a laboratory-built analogue-to-digital converter, linked to a BBC model B microcomputer. Individual voltage-

clamp experimental protocols are described in appropriate sections of the text.

2.3. Data Capture

Current- and voltage- clamp data were recorded on magnetic tape (Ampex) using a RACAL Store-7D FM-recorder. Data were played back subsequently and, for some experiments, individual records photographed from the oscilloscope screen using a Shackman oscilloscope camera. Kodak Kodalith ortho film type 3 (35mm) was used and developed with Ilford PQ Universal developer according to APPENDIX IV, under Rattan 1B safelight. Other data were downloaded using a Gould Colorwriter 6120 plotter, interfaced to the oscilloscope *via* a RS423 interface, and a chart recorder (Gould Recorder-220) connected to output-channels of the Racal recorder. Measurements were taken from individual records displayed on the oscilloscope using a Gould 160 waveform processor connected to the oscilloscope. Using this processor it was possible to filter records and also to take accurate measurements in both X and Y planes.

2.4. Graphs

All graphical relationships were constructed on a Tandon PCA computer using 'Harvard Graphics' (Microsoft). For current-voltage relationships the line graph option was selected which gave a curve of best fit through the data points. For graphical representation of action potential burst-structure the line graph option was chosen, but, in this instance, consecutive points were connected by straight lines.

CHAPTER 3. PLATEAU POTENTIALS IN A COCKROACH MOTONEURONE

INTRODUCTION

Previous work on the excitability of motoneurone D_f has focused on the generation of sodium or calcium dependent action potentials following surgical or pharmacological interventions (Pitman, Tweedle and Cohen, 1972b; Pitman, 1975; Pitman, 1979; Pitman, 1988). Such electrical events are relatively fast, presumably as a result of relatively rapid inward and outward current activation and inward current inactivation. Because these fast, short duration responses can be elicited using comparatively brief stimulus regimens (under 100ms in duration; Pitman, 1979) information concerning the electrical properties of D_f is only detailed for events which are relatively rapid in their activation, as the time window analysed in these previous experiments was comparably narrow. Thus, little information exists concerning excitable properties which may mediate somewhat slower, longer duration responses in this cell.

This series of experiments was designed and performed to uncover any relatively slow active events which might be recorded from the normal, untreated D_f. Any such non-linear membrane properties of this motoneurone could influence the way in which the cell

responds to synaptic inputs and affect the generation of impulses in the excitable portion of the axon and, thereby, activity in the coxal depressor muscles of the hind-leg (Pearson and Iles, 1970; 1971). Since motoneurones such as D_f form 'the final common pathway' in circuits generating locomotor activity, a re-examination of the membrane properties of this cell was viewed as having potentially interesting implications not only in terms of biophysical properties of the neurone, but also in terms of information processing by cells involved in pattern generation in insects.

The results presented here will describe the activity which can be recorded from the untreated D_f: in the absence of any applied stimulation; in response to relatively short duration depolarising stimuli; and in response to longer duration depolarising stimuli. These results will demonstrate that motoneurone D_f possesses non-linear membrane properties which allow it to generate plateau potentials. Plateau potentials will be characterized in terms of: their topographical origin within D_f; their influence upon impulse generation; gross changes in membrane properties during these events (recorded under current-clamp); specialized membrane properties involved in plateau generation (recorded under voltage-clamp); ionic mechanisms involved in plateau generation. Results concerning pharmacological manipulation of plateau

potential production will be presented and evidence for non-linear properties of two other insect motoneurones will be given. These results will then be discussed.

RESULTS

3.1. Identification and characterization of plateau potentials in D_f

3.1.1. Properties of the resting D_f

The resting potential of motoneurone D_f immediately following dual impalement was seen to vary between -60mV and -80mV. The mean value of the resting potential of D_f was -70.8mV (S.E. ± 1.0, n=25). Values for mean resting potential were taken after both microelectrodes had been in place for 10-20 minutes (frequently membrane potentials at the more positive end of the above range would hyperpolarise during this time period without any concomitant fall in effective membrane resistance, presumably indicating recovery from damage incurred during impalement).

The effective membrane resistance of D_f was observed to lie between 3 and 10 MΩ (5.9 MΩ; S.E. ± 0.4, n=25); this was measured by taking the current necessary to hyperpolarise the membrane potential to a steady-state potential 10 mV more negative than the

resting potential. The membrane time-constant was 9.0ms (S.E. ± 0.7, n=9), measured from membrane potential relaxations following brief (<10 ms) hyperpolarisations of the neurone.

D_f was not observed to produce spontaneous action potentials (Fig. 3.1). Some preparations were entirely quiescent in the absence of injected current; for such cells recordings taken at the resting membrane potential were devoid of any spontaneous activity (Fig. 3.1A). In some preparations synaptic activity was in evidence: small amplitude excitatory and, less frequently, inhibitory post-synaptic potentials could be recorded (Figs. 3.1B and 3.1C respectively).

3.1.2. Response to short duration depolarising pulses

The response of the normal D_f to short duration, depolarising current pulses is shown in Fig. 3.2. At low levels of injected current the cell responded passively, essentially in the same manner as a simple resistance-capacitance network; the responses to symmetrical, low amplitude pulses had similar magnitudes and time courses (not shown). With larger magnitude current injection the cell exhibited a series of damped membrane oscillations, the amplitude of which decrement throughout the applied pulse. The magnitude of the oscillatory response depended on the magnitude of the injected current, thus it is not all-

or-none. Neither does it outlive the duration of current pulse which evokes it, rather the membrane potential drops (at a rate determined by the time constant of the membrane) rapidly back to the resting level (Pitman, 1979).

3.1.3. Non-linear membrane potential responses recorded from the normal D_f

In initial experiments D_f was challenged with 200-300ms depolarising pulses of increasing amplitude and the resultant changes in membrane potential measured. In six of eleven experiments, neurones responded to increasing current steps in a non-linear manner. Fig. 3.3A shows the response of a cell to four levels of depolarising current. In such neurones, sustained (DC), depolarising current usually evoked a series of active depolarising events which were superimposed on the steady-state shift in membrane potential induced by the DC current (Fig. 3.3B). Such responses were characterised by a sharp inflection on the rising phase, a plateau phase, an afterhyperpolarisation following each event and a strong afterhyperpolarisation following cessation of the depolarising stimulus. This is clearly visible in Fig 3.3B, where an afterhyperpolarisation took the membrane potential of the cell beyond -85mV (-80mV is marked by a dotted line in this record).

FIGURE 3.1: Recordings taken from D_f in the absence of injected current. (A) Quiescent neurone. (B) Small amplitude depolarisations from a second neurone resulting from excitatory synaptic input: asterisk marks a small event which is probably a single EPSP; the larger events (marked with arrows) probably result from stronger excitatory drive. (Ci) Rapid downward deflections on this record, taken from a third neurone, result from inhibitory synaptic input; two IPSPs are shown at a faster time-base (Cii). Resting potentials (A)-75mV; (B)-71mV; (C) -70mV.

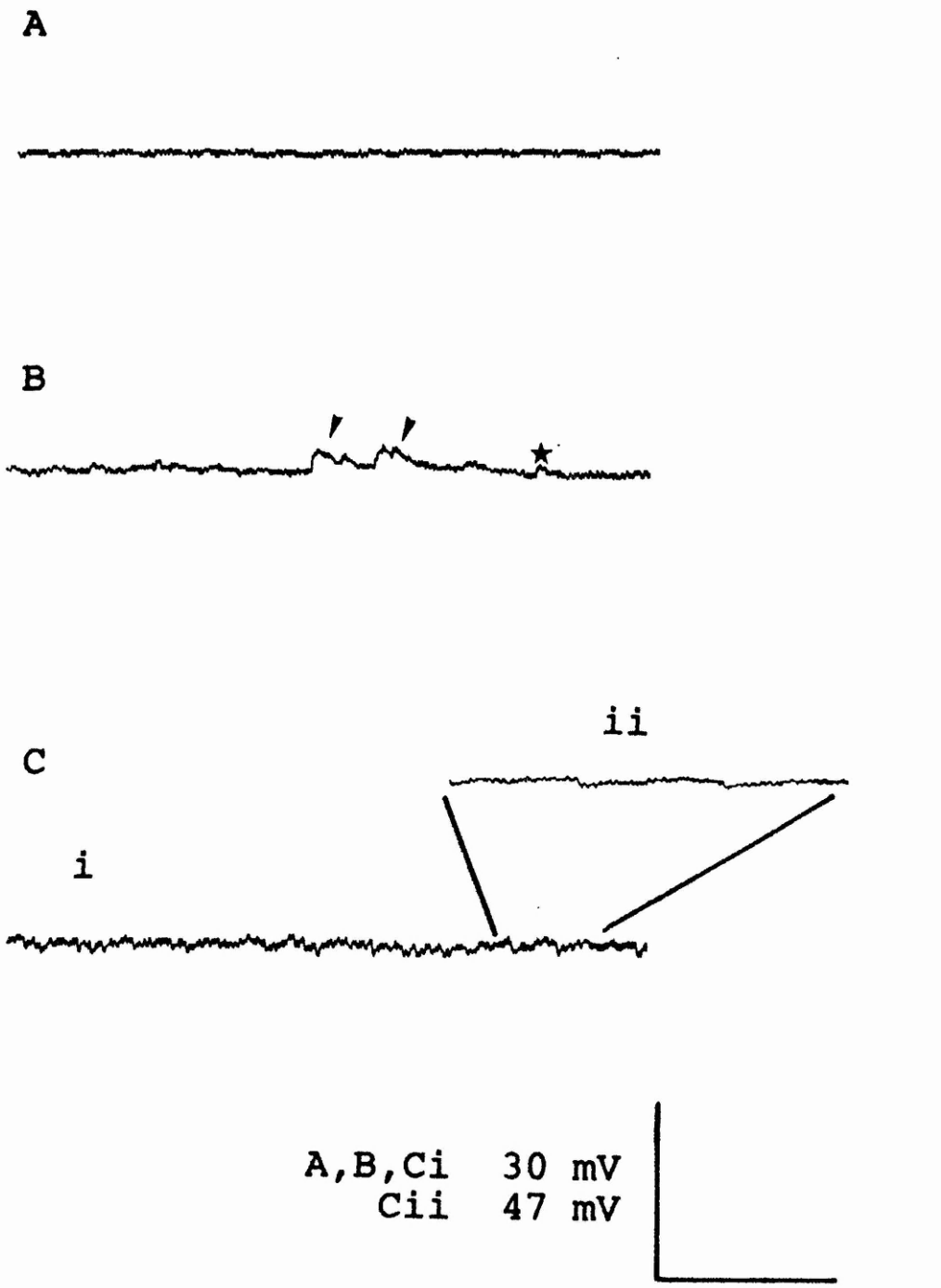

A
B
C
ii
i
A,B,Ci 30 mV
Cii 47 mV
A,B,Ci 1.5 s
Cii 470 ms

FIGURE 3.2: Graded oscillatory response of D_f to short duration depolarising current pulses of increasing amplitude (A-D). Resting potential of cell -68mV. Horizontal bars represent duration of applied depolarisations.

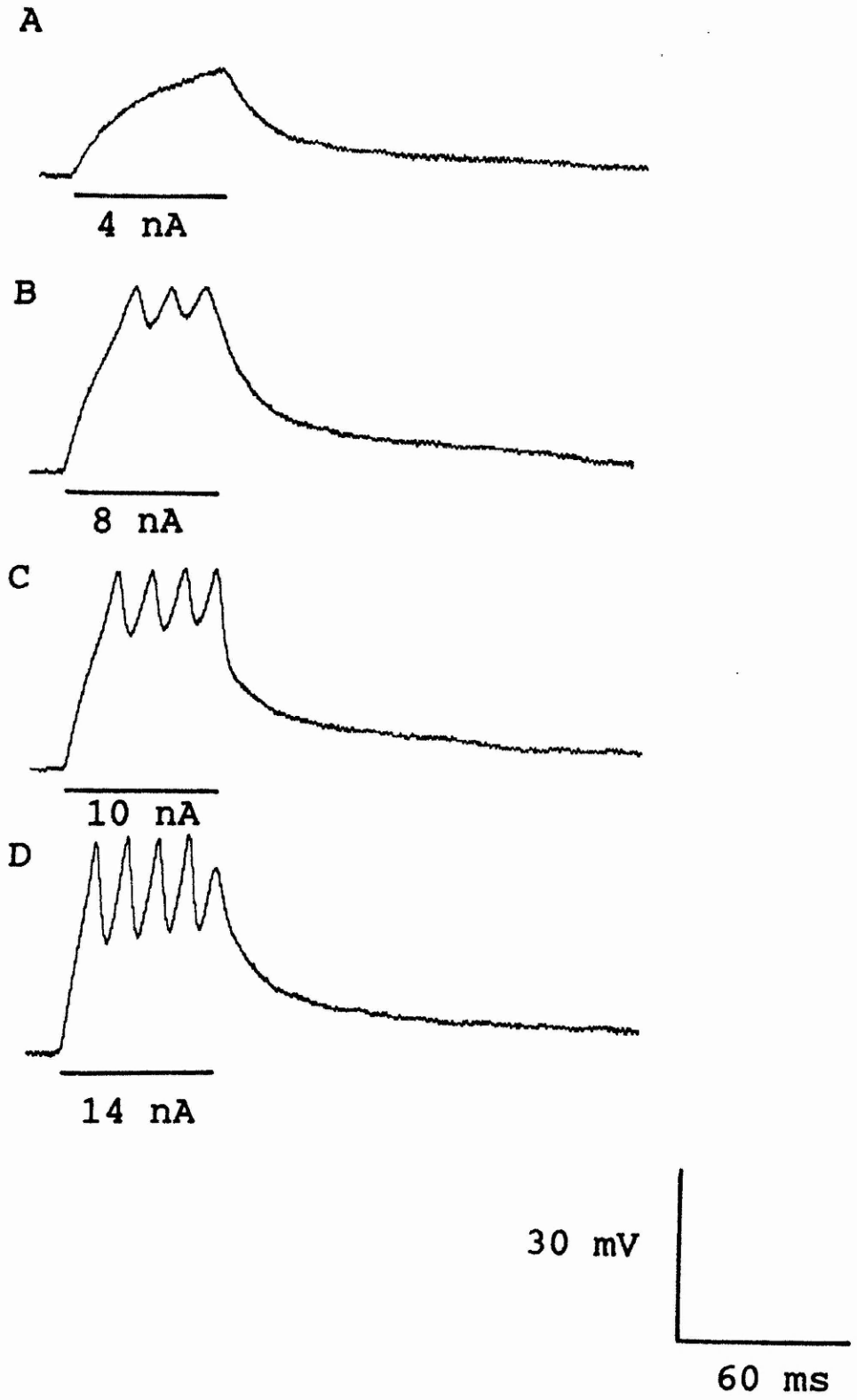
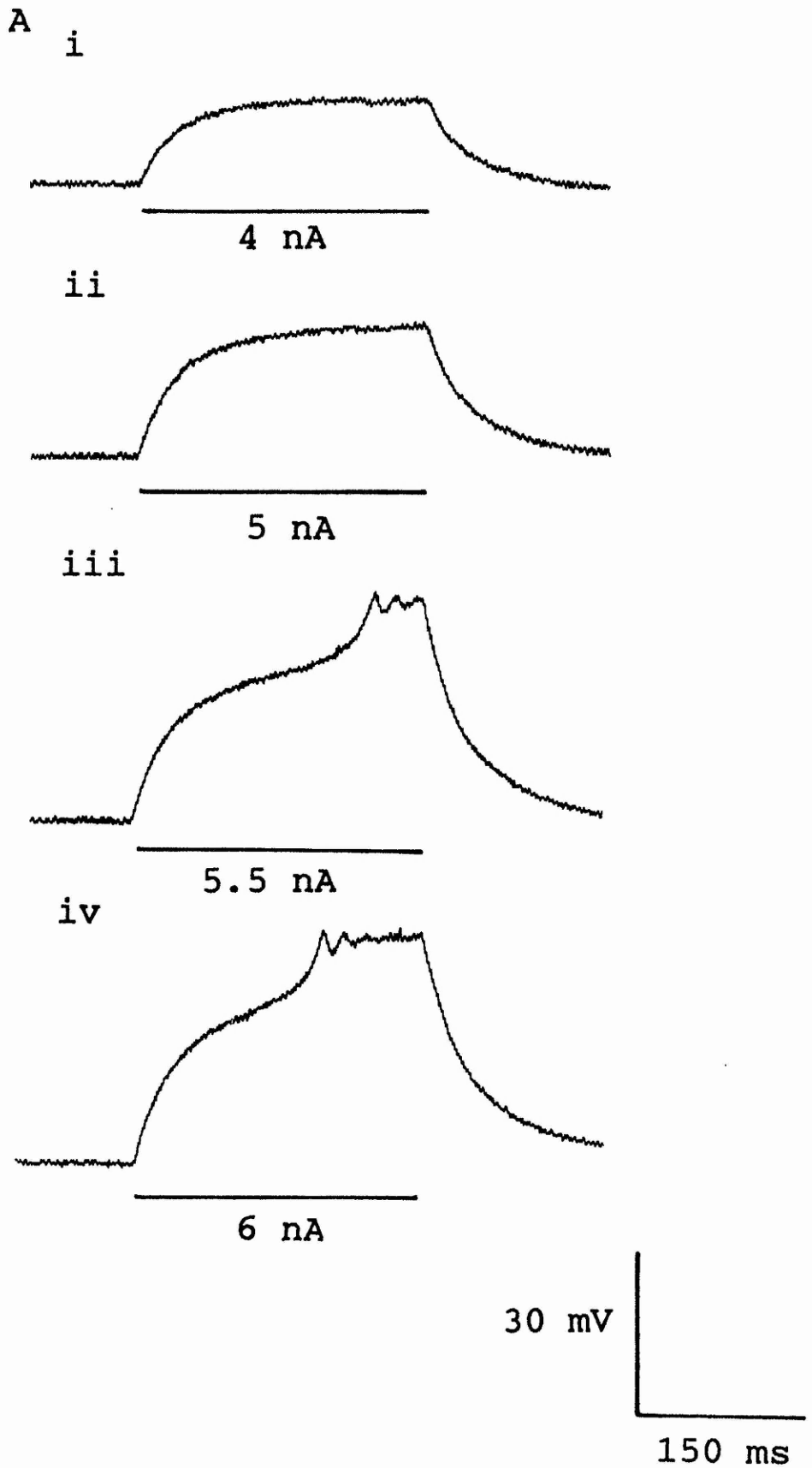
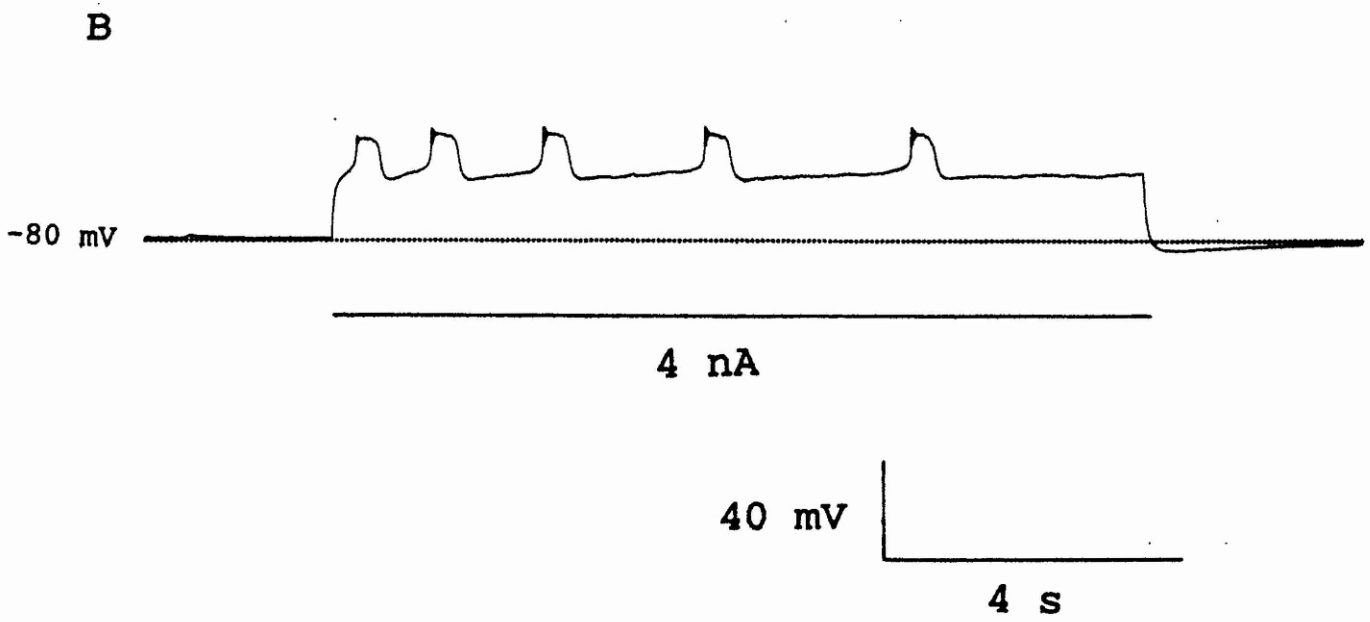

A
4 nA
B
8 nA
C
10 nA
D
14 nA
30 mV
60 ms

FIGURE 3.3: (A) (opposite) Non-linear response of D_f to 200-300 ms depolarising current pulses of increasing amplitude (i-iv).

(B) (next page) Response of same cell as (A) to a sustained depolarisation lasting several seconds. A reference potential (-80mV) is represented by a dotted line.

Resting potential of cell -78mV. Horizontal bars represent duration of applied depolarisations.


A
i
4 nA
ii
5 nA
iii
5.5 nA
iv
6 nA
30 mV
150 ms


B
-80 mV
4 nA
40 mV
4 s

3.1.4. Plateau potentials recorded from D_f

In further experiments it was established that long duration depolarising current pulses (between 500ms and 2s depending on the preparation) could evoke active membrane responses which differed markedly from the response of the cell to short duration pulses (Fig. 3.4). Short duration pulses evoked a series of graded, damped membrane oscillations which lasted merely as long as the applied depolarisation (Fig. 3.4A). In contrast, long duration pulses could evoke active membrane responses which frequently outlived the duration of the applied stimulus (Fig. 3.4B); this indicates that such events were regenerative. These events could not be elicited by short duration pulses, but were selectively activated by longer duration stimuli, indicating that these events are relatively slow in their activation. These regenerative, active membrane responses possessed a distinct threshold, were characterised by an inflection on their rising phase (indicating the active nature of the event) and a plateau phase at the peak of the response. The threshold level for activation of such responses lay within the potential range from -60 to -40 mV (-51.3 ± 0.8mV; mean ± SEM; n=23). The current threshold for activation of these responses was considerably smaller than that for activation of damped membrane oscillations by shorter duration depolarisations. In most, but not all, instances the plateau phase of the

event was surmounted by a series of attenuated action potentials (4 to 8 mV). The duration of these action potential series varied with the duration of the plateau phase.

Plateau potentials recorded from D_f were generally of relatively long duration; for a sample of 22 cells the mean plateau duration was 1.5 ± 0.1s (mean ± SEM), although in a number of experiments plateau durations outside of this range were recorded. Durations were taken at the half-height of the event as measured from the inflection on the rising phase of the response. Plateau potentials possessed a relatively slow repolarisation phase, compared to the rising phase. This repolarisation was biphasic: it usually included a prolonged afterhyperpolarisation (Fig. 3.5), which in some instances approached 1 minute in duration. During this afterhyperpolarisation, application of current pulses of similar magnitude to the initial suprathreshold stimulus failed to produce plateau responses (Fig. 3.5). During the afterhyperpolarisation, therefore, the threshold stimulus was raised, in effect making the neurone relatively refractory to a constant amplitude driving input until a critical level of recovery from the afterhyperpolarisation had been reached.

FIGURE 3.4: Differential response of D_f to short duration (A) and long duration (B) depolarising current pulses. (Ai-iii) Short pulses of increasing amplitude produced a graded oscillatory response. (Bi-iii) Longer duration pulses could evoke a plateau potential with a distinct threshold; a reference potential (-70mV) is represented by a dotted line. Cell resting potential -70mV. Horizontal bars represent duration of applied current pulses

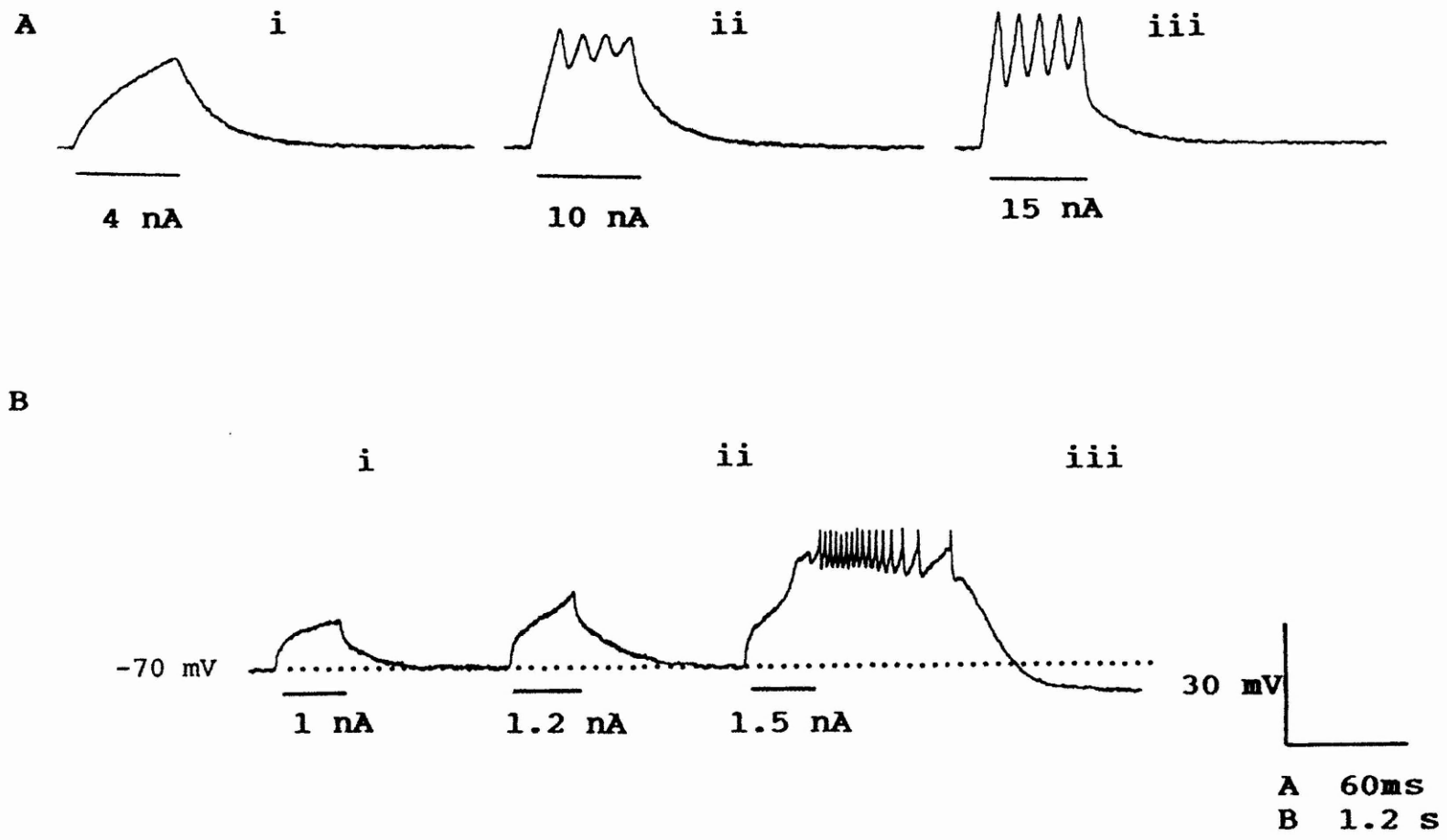
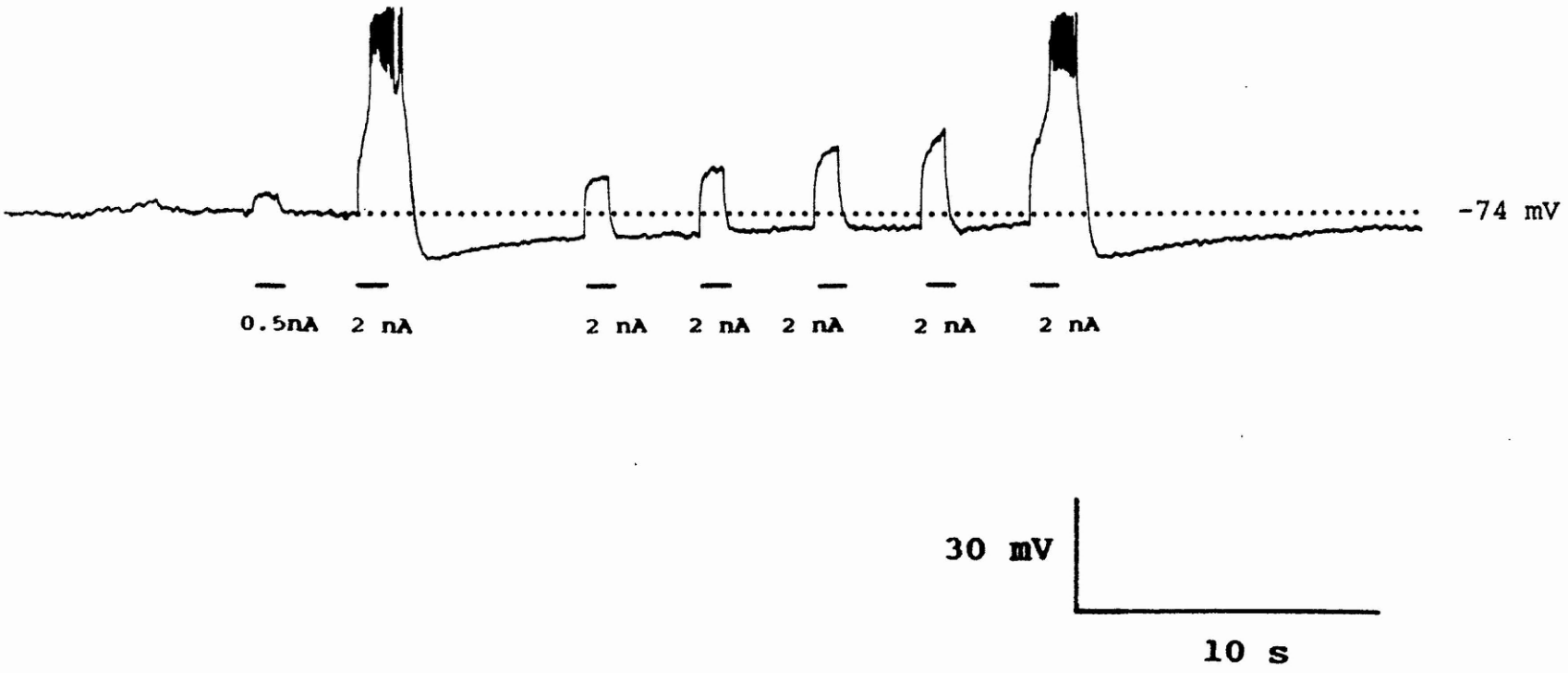

A
i
ii
iii
4 nA
10 nA
15 nA
B
i
ii
iii
-70 mV
1 nA
1.2 nA
1.5 nA
30 mV
A 60ms
B 1.2 s

FIGURE 3.5: Plateau potentials in D_f are followed by a prolonged afterhyperpolarisation. After receiving a suprathreshold depolarisation, this cell could not produce a second plateau response to similar depolarising pulses until nearly twenty seconds had elapsed. A reference potential (-74mV) is represented by a dotted line. Resting potential -74mV. Horizontal bars represent duration of applied current pulses.


-74 mV
0.5nA
2 nA
2 nA
2 nA
2 nA
2 nA
2 nA
30 mV
10 s

3.1.5 Plateau potentials in D_f drive action potentials

As illustrated in Figs. 3.4B and 3.5 plateau potentials were capable of driving the neurone into generating action potential bursts which were recorded in the soma as a series of small amplitude action potentials surmounting the crest of the plateau-response. Some neurones did not exhibit spikes on the crest of the plateau phase. In general terms, neurones appeared more likely to generate plateau potentials and the plateau response appeared more likely to be surmounted by attenuated spikes if impalement of the cell had been performed relatively cleanly. This suggests that the plateau response is far more susceptible to insults to the integrity of the neurone than, for example, the damped membrane oscillations elicited by larger amplitude, shorter duration current pulses since these could be recorded from neurones even after considerable damage.

The action potentials driven by plateau potentials were very different in their appearance to the somatic action potentials which can be recorded from D_f following anoxia, or citrate-anion injection (Pitman, 1979; 1988). In particular, they were of much smaller amplitude and possessed smaller afterhyperpolarisations than the action potentials which could be recorded following these treatments.

(This is illustrated in Fig. 3.6; citrate- or anoxia-induced action potentials were obtained by following protocols outlined in chapter 2, sections 2.1 and 2.2.6). For the amplitude of these spikes to be so attenuated (4-8mV) it follows that their site of origin must be some distance away from the recording site.

In some experiments (n=4) the axonal activity of D_f was monitored using a suction electrode applied to N5 (see methods section) whilst the soma was driven to generate plateau potentials using depolarising DC current injection. In this way it was possible to monitor the intracellular somatic and extracellular axonal responses simultaneously. Fig. 3.7 shows simultaneous extra-and intracellular recordings during a plateau potential. The extracellular recording reveals that axon spikes correlate with somatically recorded spikes on a 1:1 basis (Fig.3.7A_{i-ii}). Fig. 3.7B is an enlarged portion of the recording from Fig. 3.7A and demonstrates that the spikes recorded in the axon precede those recorded in the soma; these spikes reach the extracellular recording site more quickly than that in the cell body. This strongly indicates that the spikes which surmount the crest of the plateau-response originate within the axon of the neurone.

FIGURE 3.6: Action potentials driven by plateau potentials in D_f differ from those which can be recorded from the soma following citrate injection or prior exposure of the animal to a period of anoxia. (A) Attenuated action potentials driven by a plateau potential in an untreated neurone. (B) Action potential recorded from a second neurone following ten minutes of citrate-anion injection. (C) Action potential recorded from a third neurone fourty-eight hours following exposure of the animal to two hours of anoxia. Dotted line in (A) represents a reference potential; resting potential of cell -70mV. Dotted lines in (B) and (C) represent resting potentials of these cells. Horizontal bars represent durations of applied depolarising pulses.

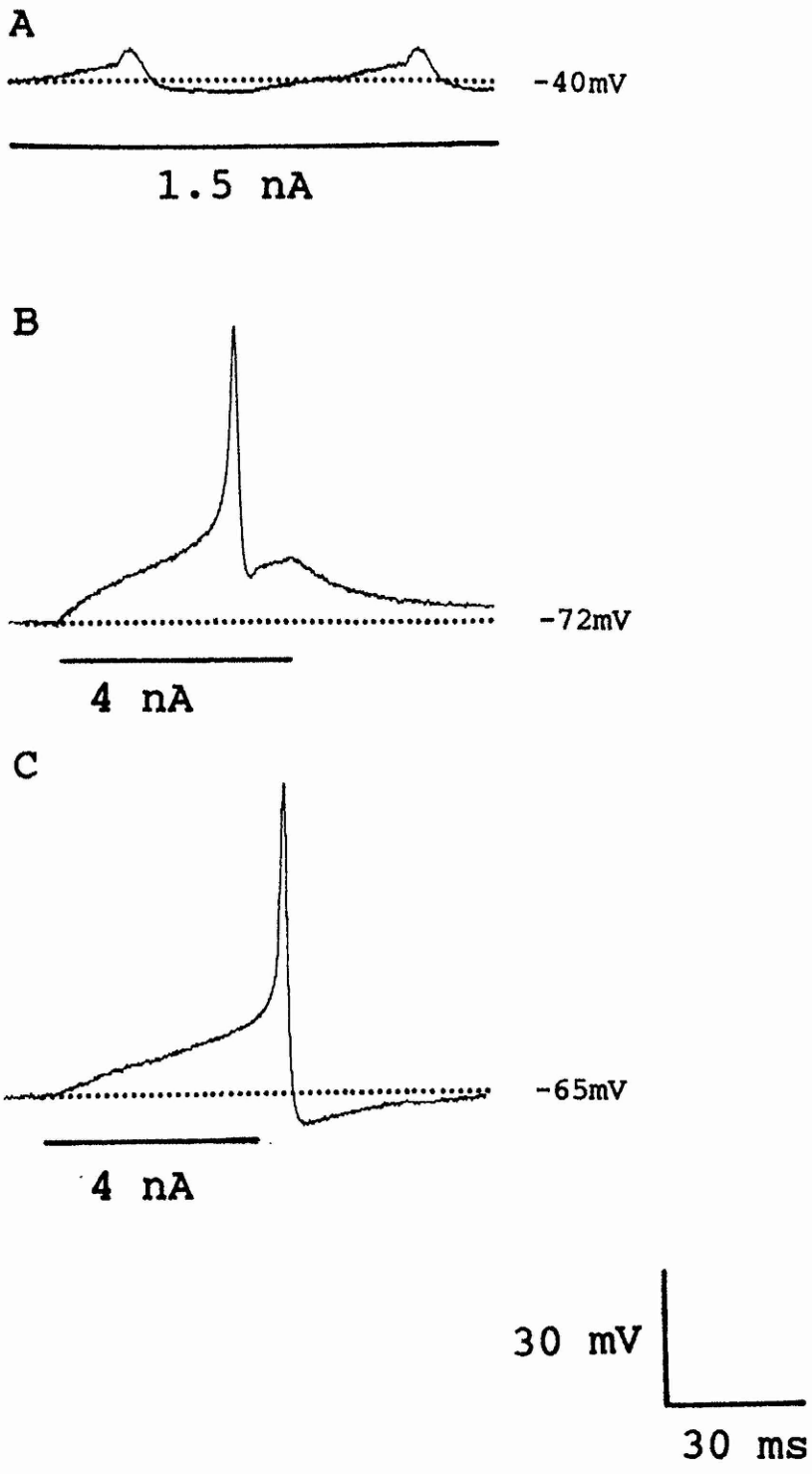
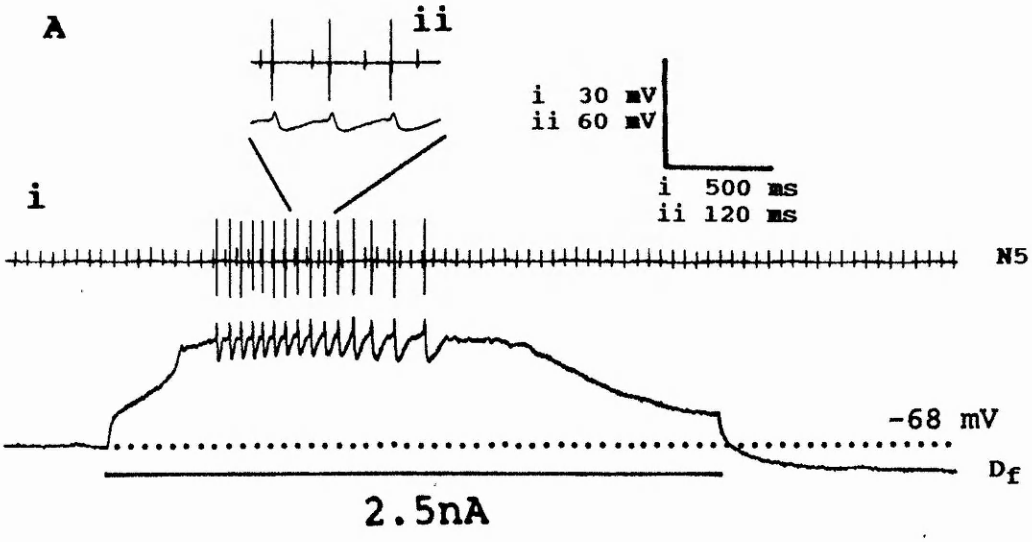
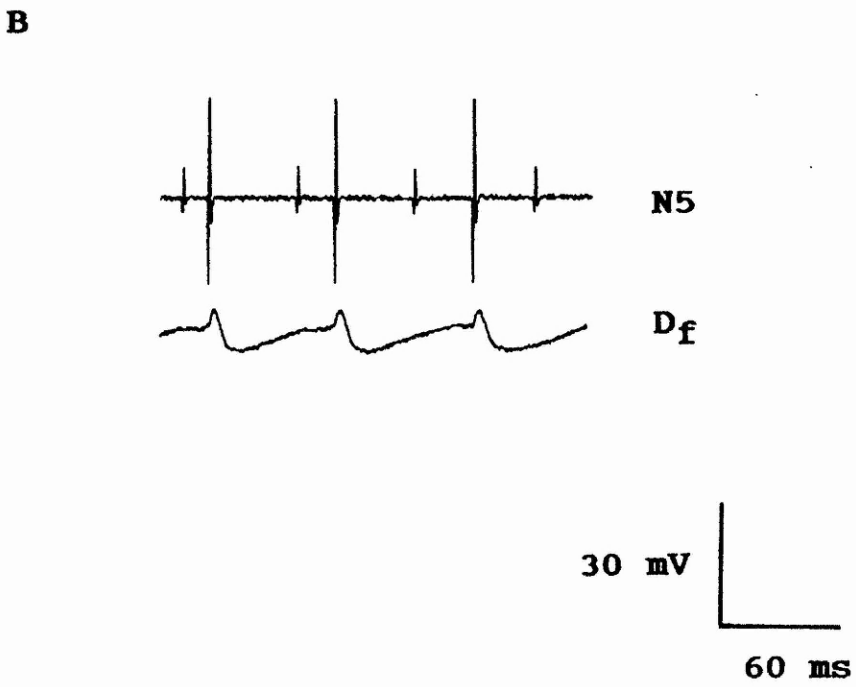

A
-40mV
1.5 nA
B
-72mV
4 nA
C
-65mV
4 nA
30 mV
30 ms

FIGURE 3.7: (Ai) Simultaneous recordings of extracellular axonal spikes (upper trace) in nerve 5 (N5) and of intracellular events in the soma of D_f (lower trace). Attenuated spikes superimposed on a plateau correlate 1:1 with axonal spikes. A portion of this record shown on an expanded scale (Aii) shows that axonal spikes shortly precede the attenuated spikes recorded in the soma. This is shown more clearly in (B). Resting potential of cell -68mV (marked in Ai with dotted line). Horizontal bar in (Ai) represents duration of depolarising current pulse.


A
ii
i
i 30 mV
ii 60 mV
i 500 ms
ii 120 ms
N5
-68 mV
Df
2.5nA


B
N5
Df
30 mV
60 ms

For some cells (n=5) the spike-burst at the crest of the plateau-response was quantified. Measurements of mean number of spikes per burst would divulge little information concerning the actual shape of the burst. Therefore, graphical analysis was performed in which instantaneous spike frequency was plotted (as the ordinate) against the order of each spike within a burst (as abscissa). The reference point for calculating instantaneous frequency (the reciprocal of the inter-spike interval) was taken as the first spike within each burst.

The graphical representation of burst-structure for two different neurones exhibiting short and long plateau potentials is given in Fig. 3.8. For each cell the action potential burst was represented as an asymmetric envelope. The spike frequency increased rapidly during the first few action potentials of the burst to a peak level and then gradually declined throughout the spike series. This decline accelerated somewhat towards the end of each series, causing a clear asymmetry of the frequency relationship. The shape of the frequency-spike relationship was similar for all neurones examined, although there were quantitative differences in absolute frequencies and slopes of the relationship between cells.

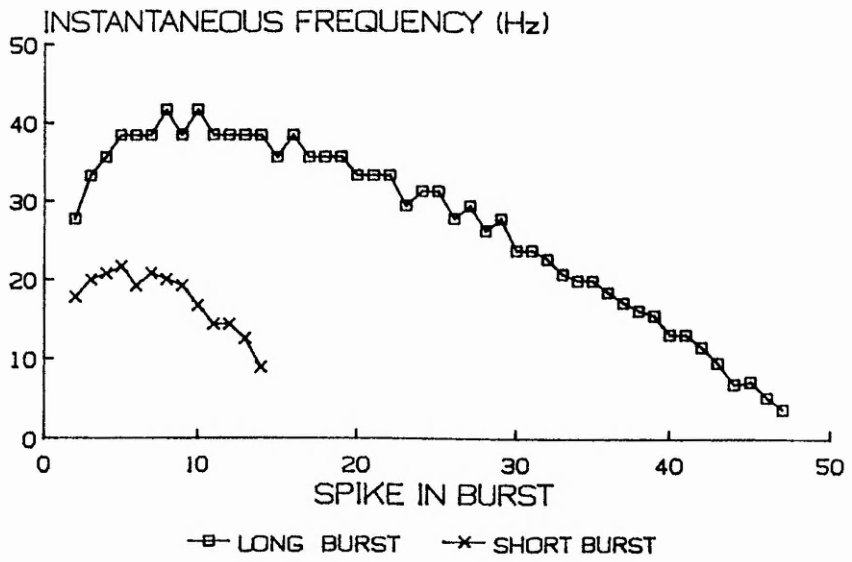


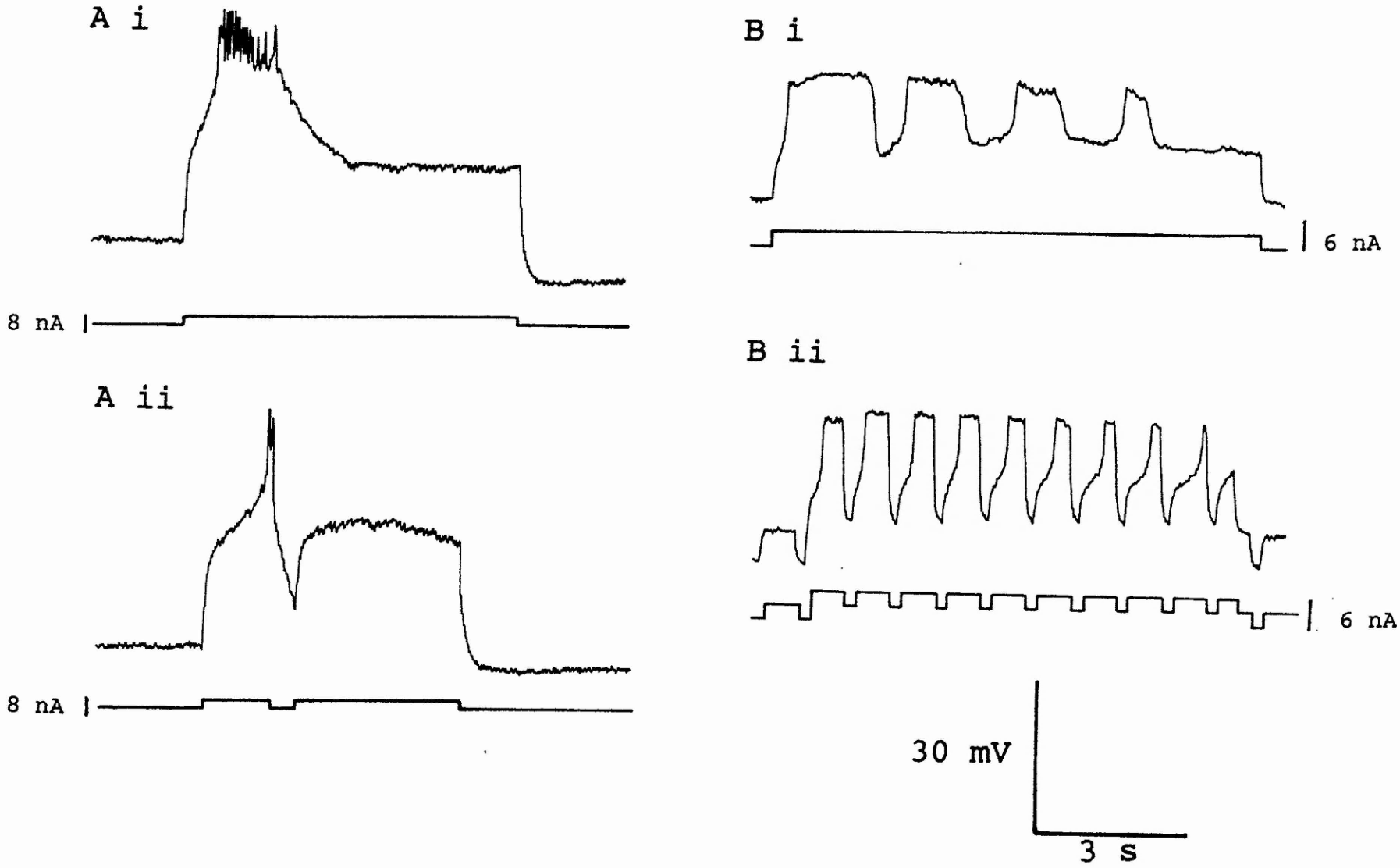
FIGURE 3.8: Graphical representation of the action potential burst driven by plateau potentials in D_f. Frequency-spike relationships are given for two neurones exhibiting long duration (open squares) and relatively short duration (crosses) impulse-bursts.

3.1.6. Premature termination of plateaux with hyperpolarising current

The plateau-response could be prematurely terminated by the injection of a sufficiently large hyperpolarising current pulse during the plateau phase of the response. For cells in which plateaux were surmounted by action potential series, premature cessation of the plateau response in turn foreshortened the spike burst (Fig 3.9A).

In some experiments (n=6) regular, hyperpolarising pulses were applied during a plateau response evoked by a sustained depolarisation. In these experiments the plateau response was interrupted by the regular hyperpolarisations; however, if the magnitude or duration of these hyperpolarisations was too small to drive plateau potentials into quiescence the maintained DC drive was sufficient to activate a plateau response at the end of each hyperpolarising pulse. In this way such hyperpolarisations were able to entrain the plateau rhythm (Fig. 3.9B).

FIGURE 3.9: Premature termination of plateau potentials by hyperpolarising current injection. (Ai) Plateau potential (upper trace) driven by a long duration (4nA) current pulse (lower trace). (Aii) Application of a relatively brief hyperpolarising pulse (-4nA) during the plateau caused it to terminate prematurely. (Bi) A series of plateaux (upper trace) evoked in a different cell by a 3nA depolarising pulse (lower trace). (Bii) Application of a series of relatively brief pulses (-3nA) during a similar depolarisation interrupted plateaux, but also resulted in the membrane potential 'rebounding' from each hyperpolarisation into a plateau, probably because the duration of the hyperpolarising pulses was insufficient to overcome the sustained depolarisation. Resting potentials (A) -75mV; (B) -70mV.


A i
8 nA
A ii
8 nA
B i
6 nA
B ii
6 nA
30 mV
3 s

3.1.7. Topographical location of the plateau response

Experiments were performed to establish whether plateau potentials could be recorded from isolated neurone somata.

For these experiments, the neurone was mechanically isolated *in situ* (see section 2.1). The morphology of D_f is such that the cell body is separated by a length of neurite from synaptic inputs, which are restricted to areas within the ganglionic neuropile (see Fig. 3.10 for morphology of D_f). Therefore, the undercutting procedure had the effect of isolating the neurone from its synaptic inputs.

One of the criteria by which the success of the undercutting procedure was measured was the baseline activity of the resting cell. As described in section 3.1.1 synaptic activity is often evident in recordings from the intact neurone. In undercut preparations a steady baseline of membrane potential was recorded from the resting cell (Fig. 3.11A). If synaptic activity was apparent in the resting cell after surgery then the undercutting procedure was deemed to have been unsuccessful and the preparation was discarded.

Mechanically isolated somata possessed similar membrane characteristics to the intact neurone. They could generate fast, damped membrane oscillations in response to short duration depolarising pulses (Fig. 3.11B), whilst plateau potentials could be elicited by sustained depolarising current injection or relatively long duration depolarising current pulses (Fig. 3.11C_i). As in intact neurones, plateau responses were characterised by an inflection on their rising phase, a sustained plateau phase and an afterhyperpolarisation. Plateau responses from isolated somata could also be prematurely terminated by a hyperpolarising current pulse applied during the plateau phase (Fig. 3.11$C_{i\,i}$).

Plateaux recorded from isolated somata differed from those recorded from the intact preparation in two important respects. Firstly, their durations were significantly shorter (duration = 0.5 ± 0.05s; mean ± SEM; n=7). This difference was visible on casual inspection, but was confirmed by use of a two tailed Students t-test ($P < 0.001$, $t = 9.19$, D.F. =27). A form of this test was used which assumed that the two sample variances were different and unknown (Parker, 1979), since it is highly unlikely that the undercutting procedure severed the proximal axon in precisely the same location for each preparation . The second way in which plateau potentials from isolated somata differed from those recorded from intact cells was that no axon spikes were superimposed

on the crest of the plateau-response. In some instances oscillations of the membrane potential were visible at the start of the plateau-response (marked with arrow in Fig 3.11C_i). These were distinct from the axon spikes observed in the intact preparation, and more closely resembled damped membrane oscillations. The lack of superimposed axon spikes in undercut preparations is important: it indicates that the mechanical isolation procedure left the neurone soma isolated not only from the synaptic inputs to the cell, but also from the axonal spike initiating zone. Mechanically isolated somata also had larger input resistances and membrane time constants than intact neurones (input resistance = 21.8 ± 3.4 MΩ; mean ± SEM; n=12; $p<0.001$ (two-tailed t-test); time constant = 20.5 ± 2.4 ms; mean ± SEM; n=13; $p<0.001$ (two-tailed t-test)). Surgical removal of the majority of the axon and dendritic arbour presumably removes a large current-sink, thereby increasing effective membrane resistance; membrane time constant would increase with membrane resistance, since membrane time constant is a product of membrane resistance and membrane capacitance (this latter parameter would not be affected by the isolation procedure).

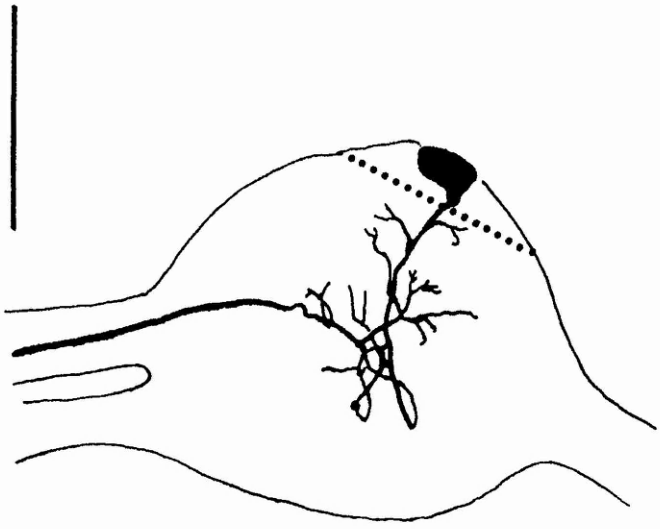
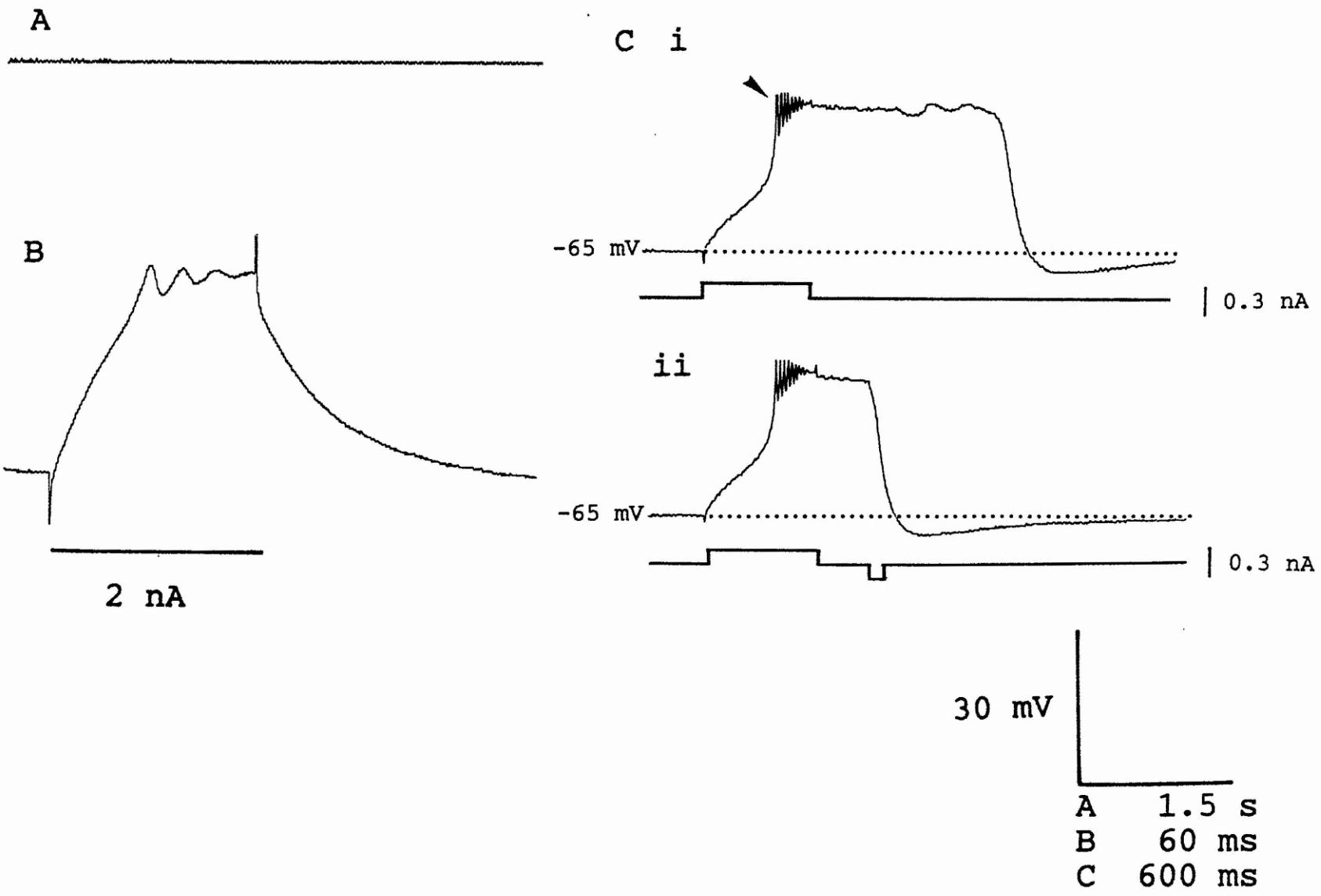


FIGURE 3.10: Drawing of the morphology of D_f, made from a photographs of a whole-mount preparation in which D_f had been stained by iontophoretic injection of cobalt into the soma. The figure shows a hemi-section of the metathoracic ganglion after this had been cut in the median sagittal plane. The cell body of D_f lies on the ventral surface (uppermost), and its axon can be seen leaving the ganglion *via* nerve 5 (left hand side of the figure). The right anterior connective is drawn to the right of the figure. Dotted line represents the line a successful incision might take in order to mechanically isolate the neuron soma. Somata which were unsuccessfully 'undercut' probably remained connected to the two short branches of the initial process which lie close to the cell body. Vertical calibration bar represents 0.5mm.

FIGURE 3.11: Recordings made from neurone somata mechanically isolated *in situ*. (A) Record of baseline membrane potential from an isolated soma; the record is devoid of spontaneous events. (B) Oscillatory response to a brief depolarising current pulse (same cell as A) applied through the recording electrode; horizontal bar represents duration of applied current pulse. (Ci) Longer duration depolarising pulses (0.15nA) applied to a different neurone evoked plateau potentials. Although these events lack superimposed, attenuated action potentials, an oscillatory component was often visible at the start of the plateau phase (marked with arrow). (Cii) Application of a brief hyperpolarising current pulse caused the plateau to terminate prematurely. Resting potentials (A, B) -67mV; (C) -65mV.


A
B
2 nA
C i
-65 mV
0.3 nA
ii
-65 mV
0.3 nA
30 mV
A 1.5 s
B 60 ms
C 600 ms

3.2. Membrane properties responsible for plateau-potentials in D_f.

The membrane properties underlying plateau potential generation in D_f were investigated under current- and voltage-clamp.

3.2.1. Conductance changes during plateau potentials

Current-clamp recordings were made from intact neurones and mechanically isolated somata to determine changes in membrane conductance during plateau-potentials. A similar protocol was adopted to that employed in section 3.1.6: small magnitude hyperpolarising pulses were applied to the neurone whilst driving individual plateau potentials, or plateau potential series with sustained depolarising current.

In seven experiments using intact neurones conductance measurements were taken before, during and after plateau potentials evoked by DC-drive. Fig. 3.12 shows the results of one such experiment. At the resting potential, the cell responded to hyperpolarising pulses with discrete hyperpolarisations. At the crest of the plateau response such hyperpolarisations were barely discernible. This indicated that there was a large increase in membrane conductance during plateau

responses. This increase in membrane conductance was not attributable to a 'masking' effect by the action potentials surmounting the crest of the response as it was also observed during plateaux from intact neurones which did not elicit axon-spikes Fig. 3.13A.

Similar experiments were performed using mechanically isolated somata (n=3). Membrane conductance was also seen to increase during plateaux recorded from these cells (Fig. 3.13B).

The increase in conductance observed during plateau potentials indicates that these events arise as a result of the activation of channels carrying an inward current, rather than inactivation of channels carrying an outward current.

These experiments were able to yield further information concerning the ionic events occurring during various stages of plateau potentials. Although membrane conductance was very much elevated during the crest of plateau potentials, this conductance increase was not uniform throughout this phase; it was greatest during the early part of the plateau phase. Fig. 3.14. illustrates the change in conductance throughout the plateau response. The response to the first hyperpolarising pulse applied during the plateau response is barely discernible, indicating a large increase in membrane conductance relative to the resting level. The second pulse produced a membrane hyperpolarisation which was sufficient to interrupt,

but not terminate the plateau; this represents a decline in conductance relative to the first response. This observation may be interpreted as a gradual inactivation throughout the response of the inward current which generates the plateau. It is noteworthy that the membrane conductance following termination of the plateau potential was higher than that at the resting potential. Whilst this may simply reflect a change in conductance consequent upon the sustained depolarisation of the neurone, it might in part have resulted from prolonged opening of channels contributing to the repolarisation of the neurone.

FIGURE 3.12: (A) Recording of membrane potential response to regular, relatively brief hyperpolarising pulses (0.5nA; not shown) at the resting potential (-71mV) and during a plateau potential evoked by a long duration depolarising pulse (2nA; duration represented by horizontal bar). Distinct hyperpolarising responses are visible at the resting potential but not during the crest of the plateau. Portions of this recording shown on an expanded scale (B) demonstrate a considerable increase in membrane conductance during the plateau phase (Bii) relative to the resting potential (Bi). In (Bi-ii) upper traces show membrane potential, lower traces represent current.

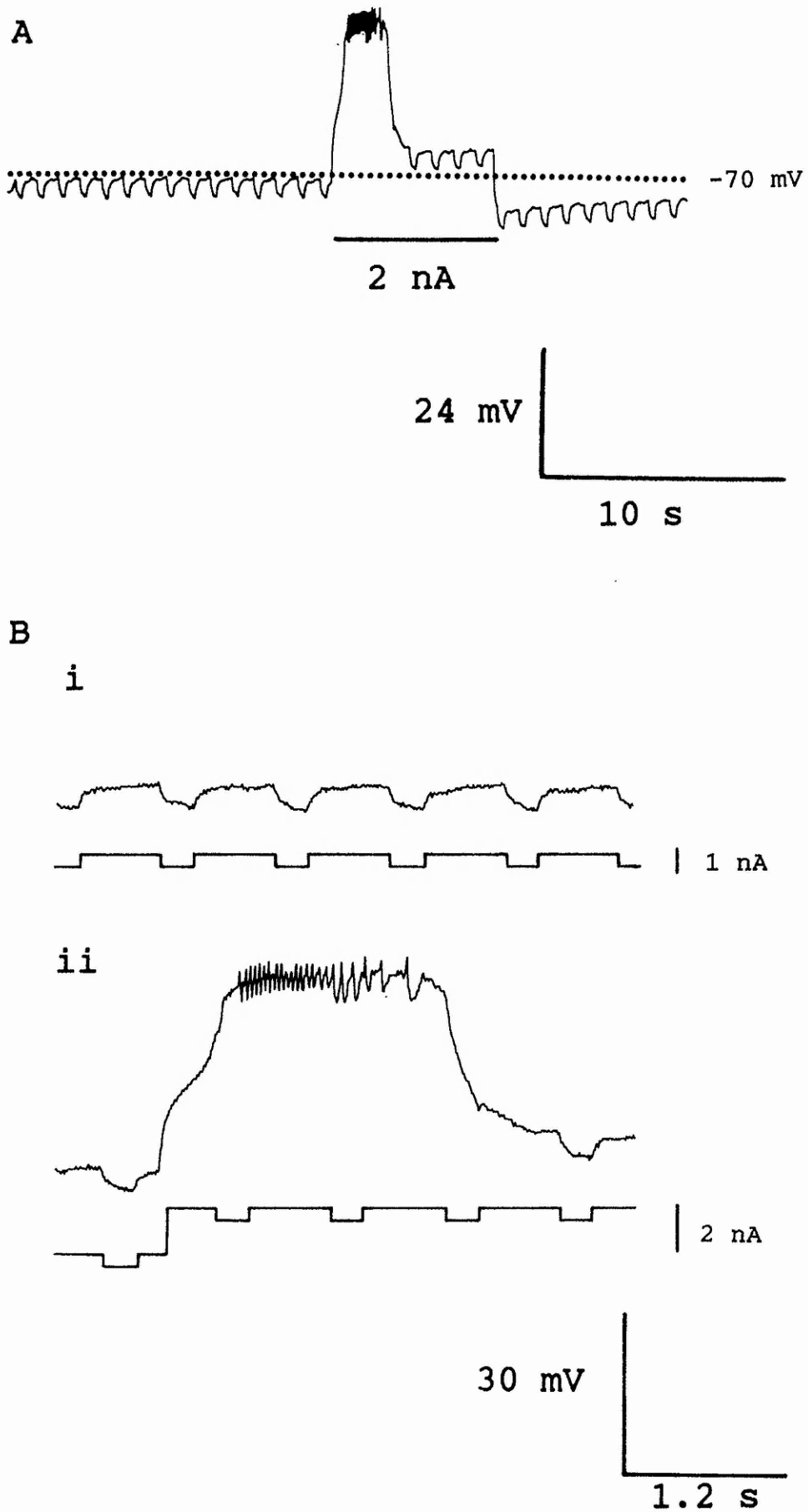

A
-70 mV
2 nA
24 mV
10 s
B
i
1 nA
ii
2 nA
30 mV
1.2 s

FIGURE 3.13 (A) A recording taken from a neurone generating plateau potentials but not axon spikes. Upper trace shows membrane potential, lower trace represents current injection. The membrane potential response to regular, relatively brief hyperpolarising current pulses is clearly visible at the resting potential (-60mV), but not during a plateau potential. This demonstrates that the increase in membrane conductance during the plateau is not associated with a masking effect of axon spikes. (B) A recording from a cell body, mechanically isolated *in situ*, also shows an increase in conductance during the plateau (upper trace -membrane potential, lower trace -applied current). During this recording a series of plateau potentials was evoked by applying a steady depolarising current to the neurone; therefore, a reference potential is shown (dotted line).

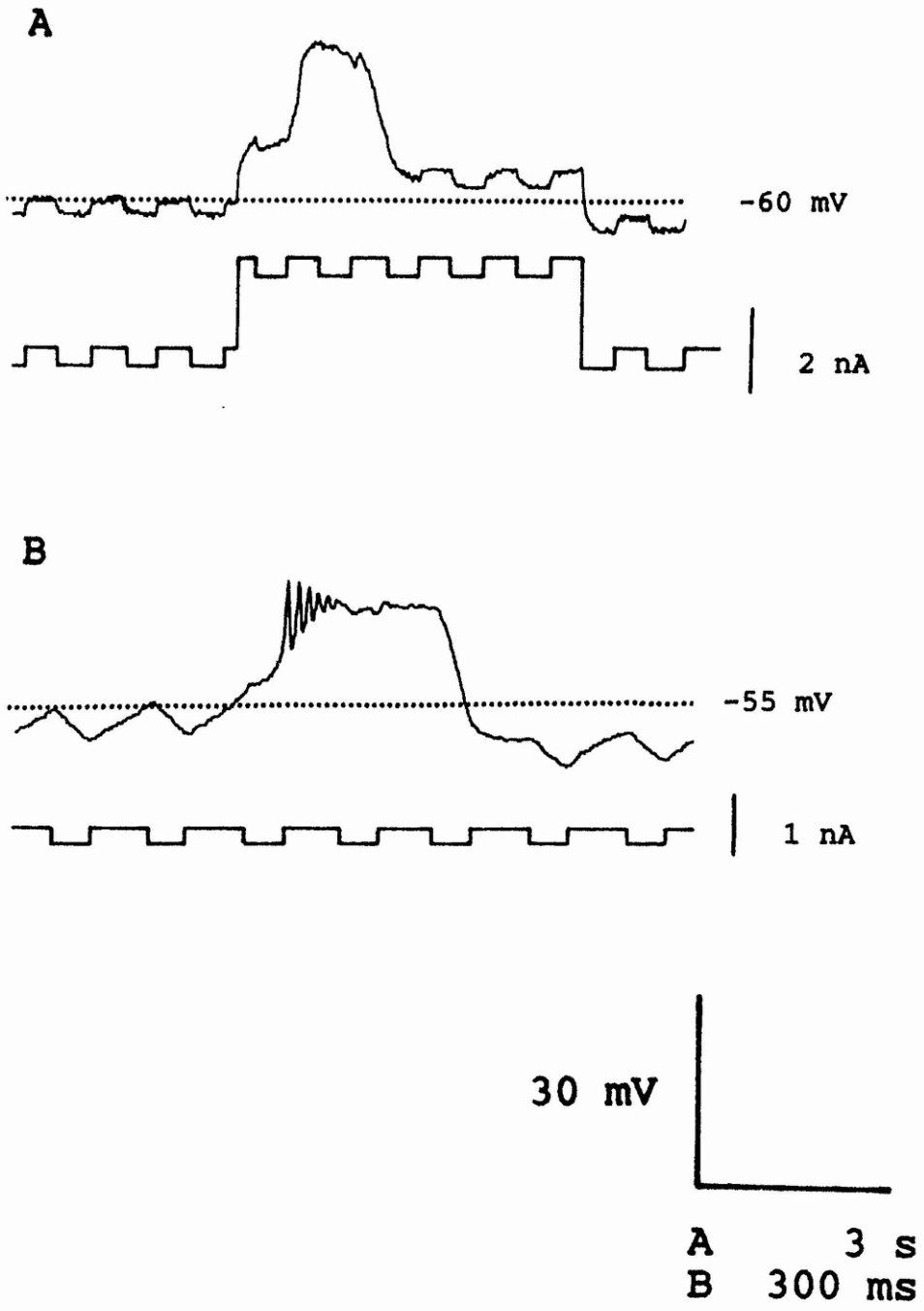
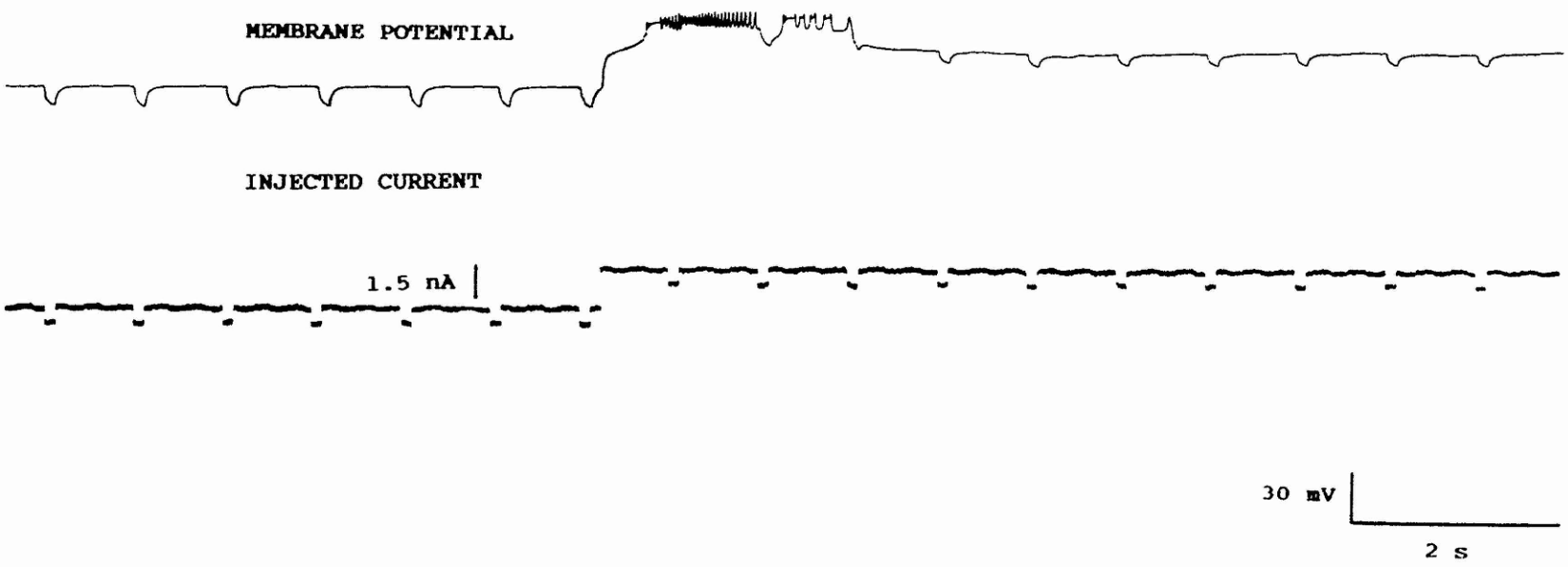

A
-60 mV
2 nA
B
-55 mV
1 nA
30 mV
A 3 s
B 300 ms

FIGURE 3.14: Record of membrane conductance measurement during a relatively long duration plateau potential. Upper trace shows membrane potential, lower trace shows injected current. The conductance increase during the plateau is not uniform; the second hyperpolarising pulse applied during the plateau produced a larger hyperpolarisation than the first. Resting potential -74mV.


MEMBRANE POTENTIAL
INJECTED CURRENT
1.5 nA
30 mV
2 s

3.2.2. Voltage-clamp

Time- and voltage- dependent membrane properties underlying plateau potential production were investigated under voltage-clamp. All voltage-clamp experiments were performed on 'intact' neurones.

3.2.2.1. Short pulse protocol

In initial experiments (n=3), cells generating plateau potentials were held at -70mV and 100ms duration successive incrementing depolarising command steps applied at a frequency of 0.5Hz. The step increment was 2mV. I-V relationships were constructed from responses sampled at 50ms after the start of each command pulse. This protocol is similar to that adopted in previous voltage-clamp experiments upon D_f (Thomas, 1984) and upon cell 3 (Nightingale and Pitman, 1988), the major difference being the relatively narrow range of membrane potentials examined in the present series (-70mV to -20mV/-30mV). Such a range was chosen since plateau potentials occur within this voltage-window. The command pulse duration was chosen to ascertain whether or not the currents underlying plateaux were evident within 100ms of suprathreshold depolarisation.

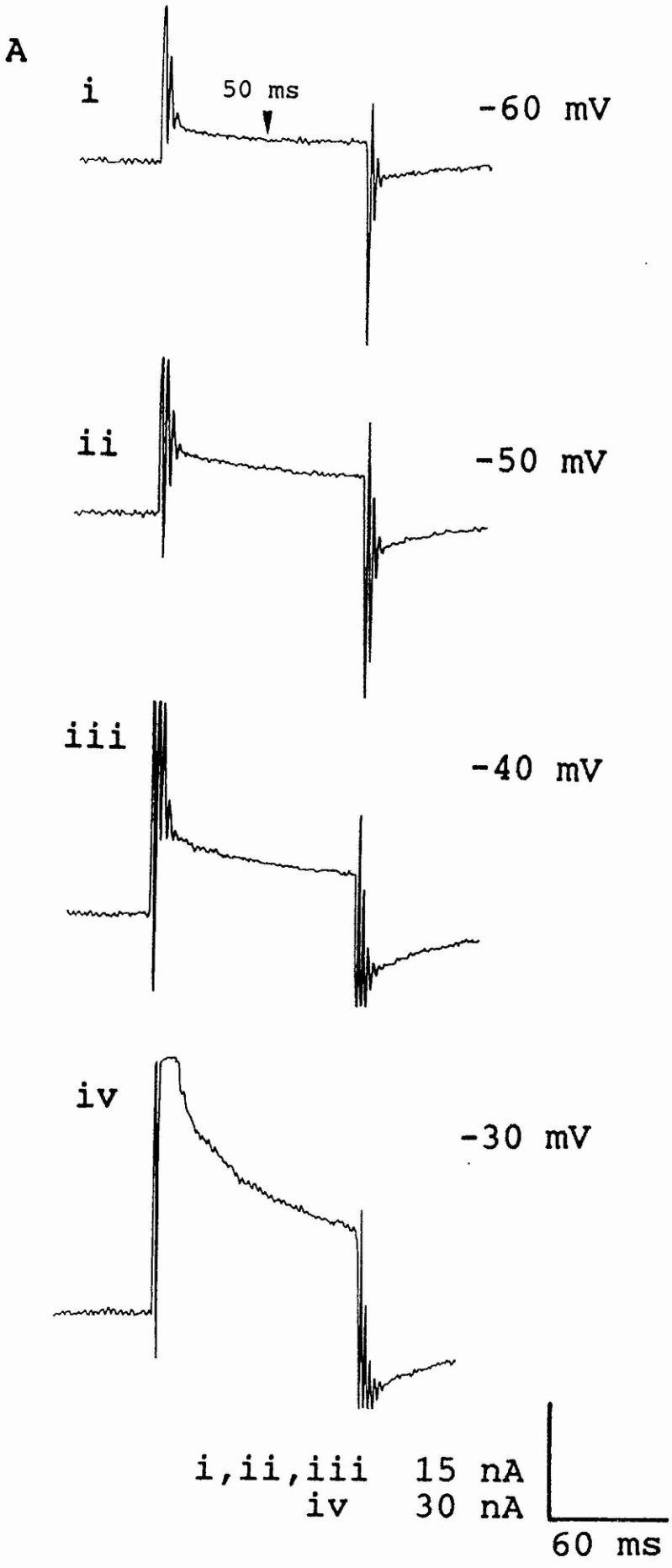
Representative current records from a typical experiment are shown in Fig 3.15A. For depolarising command steps the current responses were outwardly

directed. The outward currents generated by small command steps (up to approximately 20mV positive to the holding potential) were relatively small. Positive to between -50mV and -40mV (the precise membrane potential value varied between preparations) the outward current sampled at any given time-point in current records increased dramatically. This sharp increase in net outward current positive to approximately -40mV represents outward rectification of the membrane of the neurone and corresponds to that observed in earlier experiments on D_f (Thomas, 1984) and cell 3 (Nightingale and Pitman, 1989). Outward currents were maximal towards the beginning of current records and gradually declined throughout the response. This current 'sag' was particularly evident in current responses to more positive command steps (for example, see record at -30mV in Fig. 3.15A) and may reflect either gradual inactivation of the channels carrying the outward current, or a run-down of a transmembrane ion gradient during the pulse. Inwardly directed 'tail' currents following each current response are consistent with build-up of potassium ions in restricted extracellular spaces during command steps, causing the potassium equilibrium potential to be positive relative to the holding potential (Thomas, 1984).

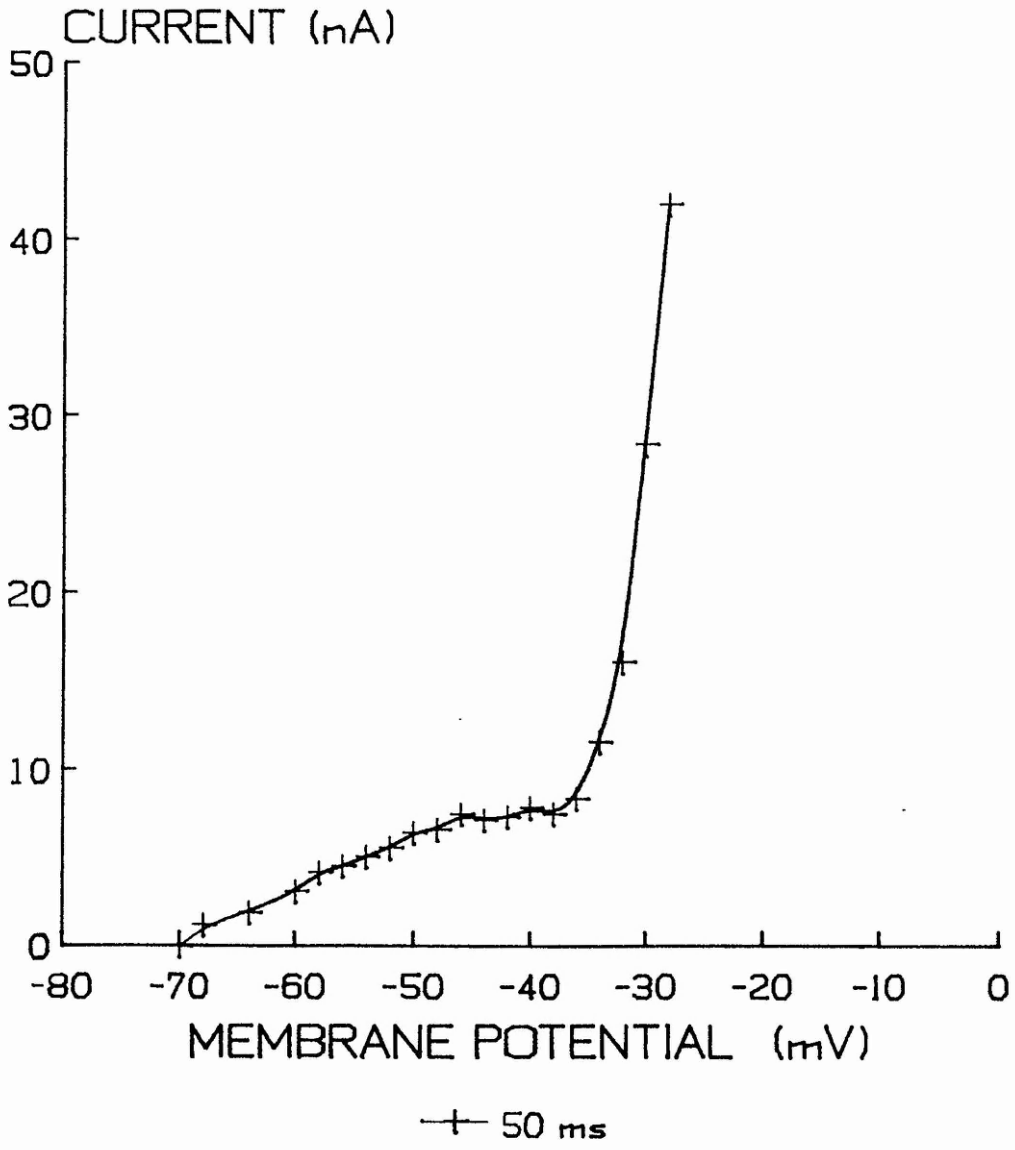
For each of the cells examined in this way the current-voltage (I-V) relationship was similar to that shown in Fig. 3.15B. I-V curves were biphasic,

showing a gradual increase in outward current to approximately -40mV and a sharp increase in outward current positive to this potential. The sharp increase in outward current at more positive potentials represents the voltage-dependent activation of outwardly rectifying channels described above. In contrast to other neurones which exhibit bistable behaviour (Wilson and Wachtel, 1974; Schwindt and Crill, 1977) no region of negative slope resistance (NSR) was apparent in the I-V relationship, despite the fact that plateaux could be evoked under current-clamp. The most likely explanation for the lack of a region of NSR in the I-V relationship is that the currents responsible for plateaux generation in D_f are relatively slow in their activation. Whilst no region of NSR was apparent in the I-V profile, some flattening of the relationship was evident over a 10-15 mV range from -60 to -40 mV (depending on the preparation). This could represent the early stages of activation of inward current(s) mediating the plateaux. However, if they were activated at all within this time-window, any voltage-dependent inward currents involved in plateau generation were not sufficiently large to overcome the dominant outward currents.

FIGURE 3.15: (A) (opposite) Net membrane currents (filtered at 556Hz) recorded from D_f under voltage-clamp. 100ms duration, depolarising command pulses (not shown) were applied from a holding potential of -70mV. Such recordings were used to construct I-V relationships for the neurone (B; overleaf). Arrow in (A) indicates point at which currents were sampled.


A
i
50 ms
-60 mV
ii
-50 mV
iii
-40 mV
iv
-30 mV
i,ii,iii 15 nA
iv 30 nA
60 ms

B


CURRENT (nA)
50
40
30
20
10
0
-80
-70
-60
-50
-40
-30
-20
-10
0
MEMBRANE POTENTIAL (mV)
50 ms

3.2.2.2. Long pulse protocol. (A negative resistance characteristic is associated with plateau production).

To identify the relatively slowly activating, voltage-dependent currents which might underlie plateau potentials the following protocol was adopted. Cells were held at -70mV and successive depolarising voltage steps applied (step increment 5mV). The duration of each voltage-jump was between 700ms and 800ms. For this series of experiments a low stimulus frequency was employed; command-pulses were applied one every minute. This stimulus regimen was used for two reasons: firstly, to take into account the relative refractoriness of the neurone observed under current-clamp during the afterhyperpolarisation following plateaux (see Fig. 3.5); secondly, to minimise the effect of transmembrane ion 'run-down' resulting from a large series of long duration command pulses applied in close succession (such 'run-down' was observed using long-pulse protocols in experiments using the cockroach metathoracic motoneurone Cell 3 (Nightingale, 1988). Refractoriness, ionic run-down, or a combination of the two could, at worst, completely obscure events of interest.

It was hoped that by applying command steps at such a low frequency, the problem of refractoriness would be eliminated and sufficient time allowed between steps to prevent significant build up of ions in restricted extracellular spaces, thereby largely

removing a primary causes of run-down in voltage-clamp experiments.

In each of twelve experiments performed upon neurones exhibiting plateau potentials under current-clamp, voltage-dependent membrane properties were identified under voltage-clamp which correlated with plateau potential generation. These properties were absent from neurones which did not exhibit plateau potentials (n=5).

Fig. 3.16 shows the current responses to command steps to -65mV, -40mV and -30mV of a D_f cell-pair from the same animal, one of which generated plateau potentials under current-clamp and one which did not. For both neurones the response at -65mV was a sustained outward current throughout the applied voltage command. However at more positive potentials the responses of the two neurones differed. For the cell in which plateau-responses were absent, successively larger command steps evoked progressively larger outward currents which, in each instance, were maximal towards the start of the response and declined throughout it. The principal difference observed for the neurone exhibiting plateaux was that in the potential range -60 to -40 mV a delayed inward-going component became superimposed on the outward current response. This is marked on the current response to a clamp potential of -40 mV in Fig. 3.16B (asterisk). This component was

not evident during the early stages of current records but became apparent at later points in the response. Positive to -40mV the delayed inward component became masked by strong outward currents, and was no longer evident. Positive to -40mV outward currents were dominant in both cells as a result of the outward rectification of the membrane described previously. The difference in properties of the two neurones was highlighted in current-voltage relationships which were constructed for each neurone from data sampled 50ms, 400ms and 600ms into current records. These are shown in Fig. 3.17.

For both neurones the I-V profile constructed at 50ms was devoid of a region of negative slope. However, for the cell generating plateaux, a region of 'flattening' of the relationship was present in the potential range -60mV to -45mV (Fig. 3.17A). This observation is similar to that made in the preceding series of experiments (Fig. 3.15). Although a region of decreased slope was present in the relationship from the contralateral cell it was far less pronounced, consistent with a difference in the active properties of the two cells (both possessed similar input resistances [5MΩm]).

At 400ms and 600ms distinct regions of negative slope resistance were observed between -55mV and -40mV in the I-V profile of the plateaux-generating neurone (Figs. 3.17B,C). Corresponding regions of NSR were absent in the I-V profile of the contralateral cell.

This finding is crucially important since it demonstrates two things: firstly, the correlation between plateau generation and a distinct region of NSR in the I-V relationship and secondly that the current(s) underlying plateau genesis in the normal D_f is (are) relatively slow in their activation. The magnitude of the NSR was greater for the I-V profile later during the current record (600ms) than earlier (400ms). It is important to realise, however, that the I-V relationships do not represent peak currents. In most instances the delayed inward component continued to develop throughout the duration of the command step and, therefore, the magnitude of the NSR region of the I-V relationship would have been larger had peak values been used for construction. However, to allow comparison between neurones generating plateaux and those which did not, discrete sample points were used . The use of discrete sample points did not unduly bias the apparent voltage-dependence of the NSR; for, whilst the delayed inward component was activated more rapidly as the membrane potential was stepped more positive, it was sufficiently slow in its activation for I-V relationships to accurately reflect voltage-dependent currents.

For both neurones exhibiting plateau potentials and those in which plateaux were absent the magnitude of the net outward currents at potentials positive to about -60mV decreased throughout the duration of

command steps (This can be seen by looking at the I-V relationships in Fig. 3.18). For example, the outward rectification positive to -40mV was smaller by up to 50% at 600ms than at 50ms into responses. This outward current 'sag' was distinct from any slowly developing inward component and was not associated with plateau potential production: it was present both in neurones exhibiting plateau-responses and those not, and may represent outward channel inactivation or potassium accumulation as discussed above.

FIGURE 3.16: Samples of currents from a D_f-pair from the same animal; one neurone did not exhibit plateau potentials (A), another did (B). Upper traces are membrane currents (filtered at 45.5Hz), lower traces show clamp potential (holding potential for each cell -70mV). Asterisk in (B) shows a developing inward current component at -40mV which is absent for the response at the same potential from cell (A). Arrows indicate points at which responses were sampled to construct I-V relationships for Figs. 3.17, 3.18.

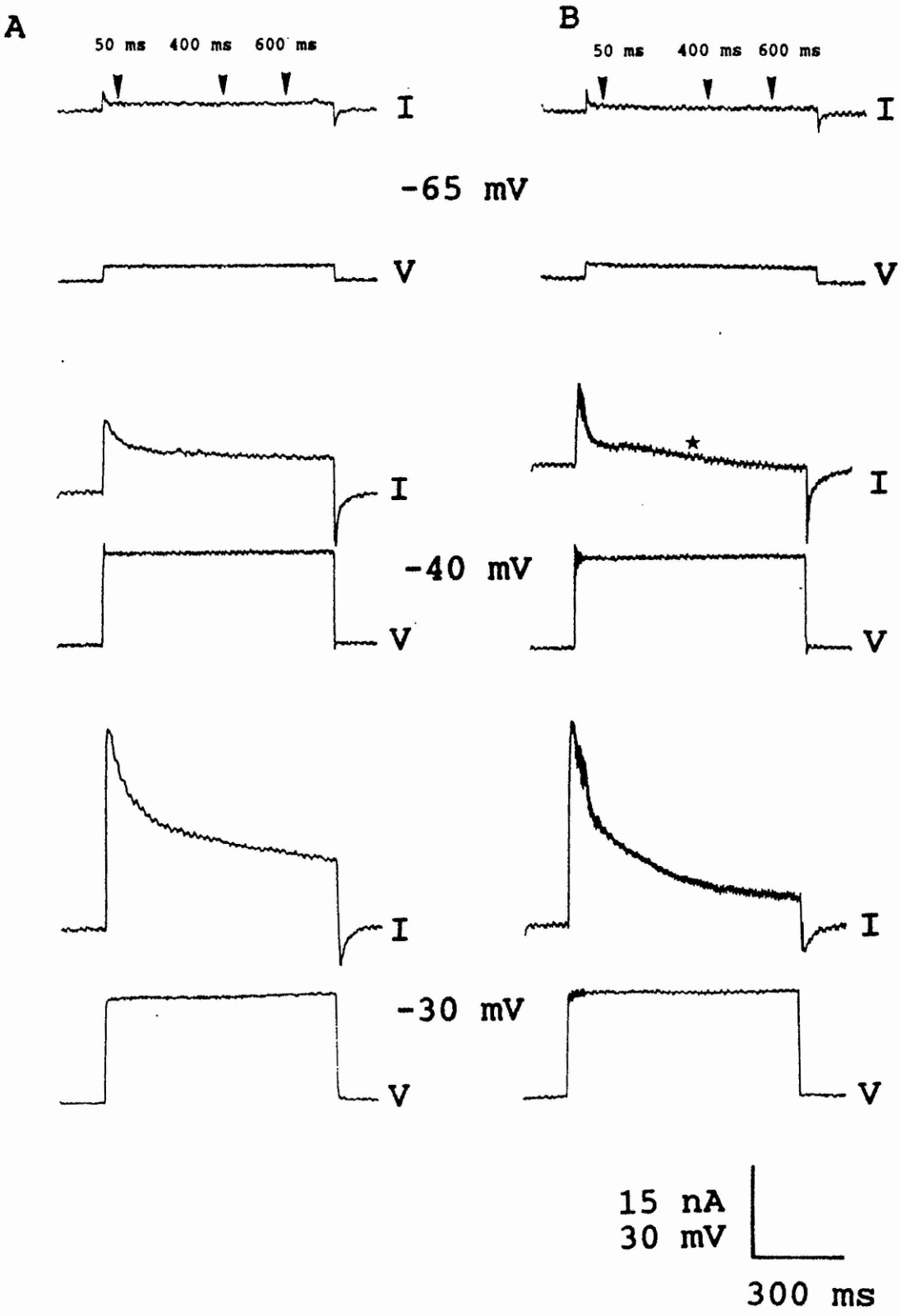

A
50 ms
400 ms
600 ms
I
-65 mV
V
I
-40 mV
V
I
-30 mV
V
B
50 ms
400 ms
600 ms
I
V
I
V
I
V
15 nA
30 mV
300 ms

FIGURE 3.17: Current-voltage (I-V) relationships for a heterolateral D_f pair from the same animal; one cell exhibited plateau potentials (solid lines in I-V curves), while the other did not (dotted lines in I-V curves). I-V relationships are from currents sampled at 50ms (A), 400ms (B) and 600ms (C) into command pulses. I-V curves from the cell generating plateau potentials show a region of negative-slope (NSR) which is absent in those from the cell which did not (B, C). However, this region of NSR was not apparent in I-V curves constructed from currents sampled early on into current responses (A).

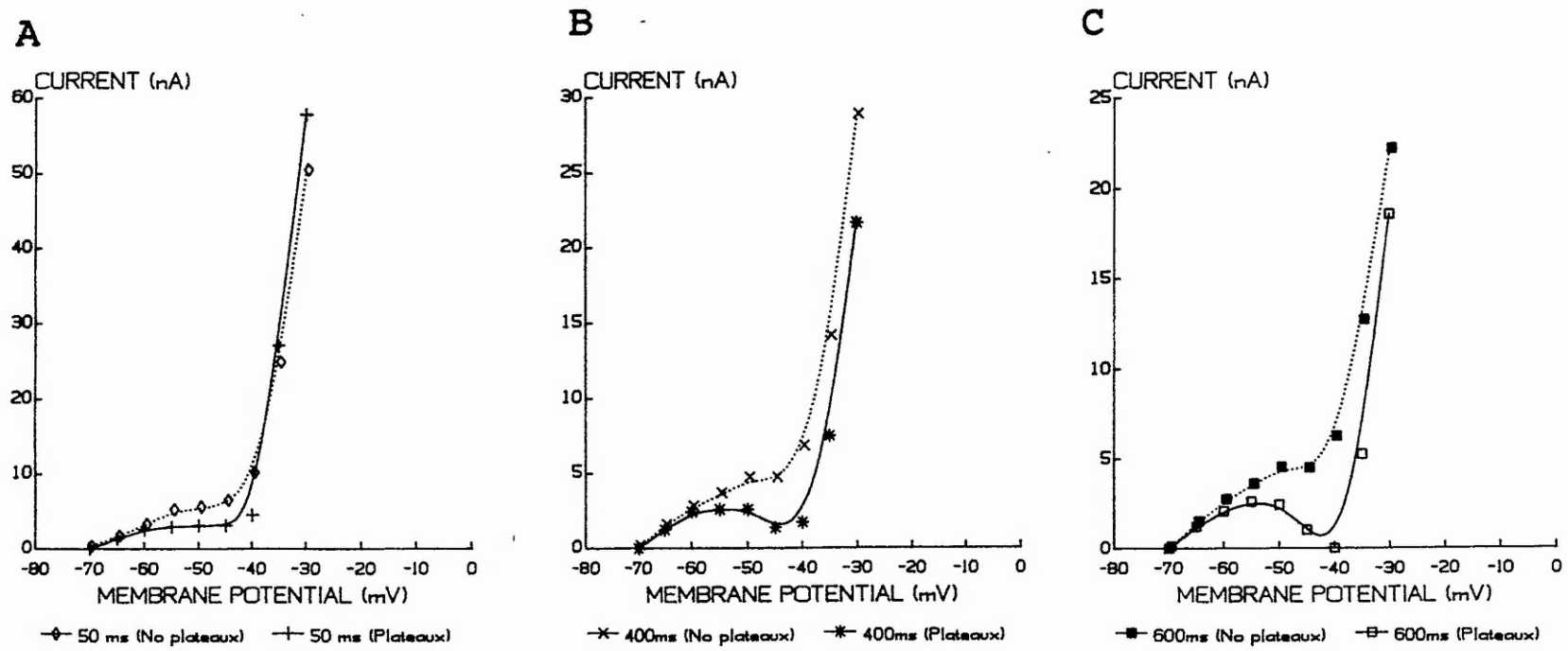
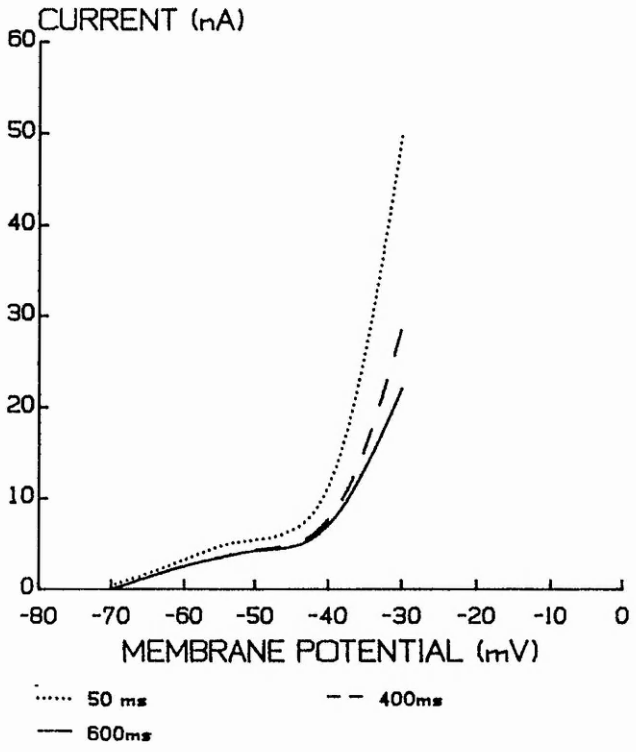
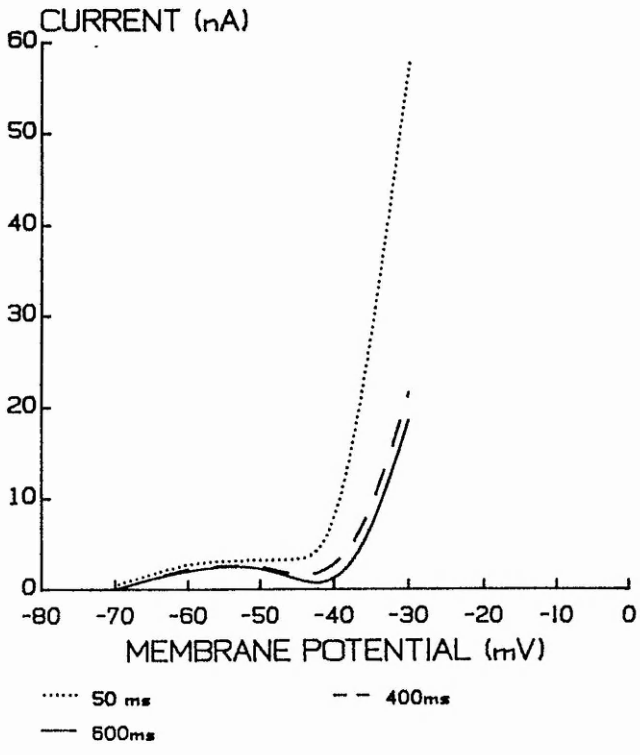

A
CURRENT (nA)
60
50
40
30
20
10
0
-80 -70 -60 -50 -40 -30 -20 -10 0
MEMBRANE POTENTIAL (mV)
50 ms (No plateaux)
50 ms (Plateaux)
B
CURRENT (nA)
30
25
20
15
10
5
0
-80 -70 -60 -50 -40 -30 -20 -10 0
MEMBRANE POTENTIAL (mV)
400ms (No plateaux)
400ms (Plateaux)
C
CURRENT (nA)
25
20
15
10
5
0
-80 -70 -60 -50 -40 -30 -20 -10 0
MEMBRANE POTENTIAL (mV)
600ms (No plateaux)
600ms (Plateaux)

FIGURE 3.18: I-V relationships for the same heterolateral cell pair as used for Figs. 3.16, 3.17 demonstrating that for any given clamp potential the magnitude of the net outward current declined throughout the current response. (A) cell which did not exhibit plateau potentials (B) cell which did exhibit plateau potentials. The decline in net outward current throughout current responses is distinct from the development of an inward current component which only occurred for cell (B); I-V relationships from currents sampled relatively late into command pulses showed a region of NSR for this neurone, but not for (A).

A


CURRENT (nA)
60
50
40
30
20
10
0
-80 -70 -60 -50 -40 -30 -20 -10 0
MEMBRANE POTENTIAL (mV)
50 ms
400ms
600ms

B


CURRENT (nA)
60
50
40
30
20
10
0
-80 -70 -60 -50 -40 -30 -20 -10 0
MEMBRANE POTENTIAL (mV)
50 ms
400ms
600ms

3.2.2.3. Evidence for inactivation of currents associated with plateaux

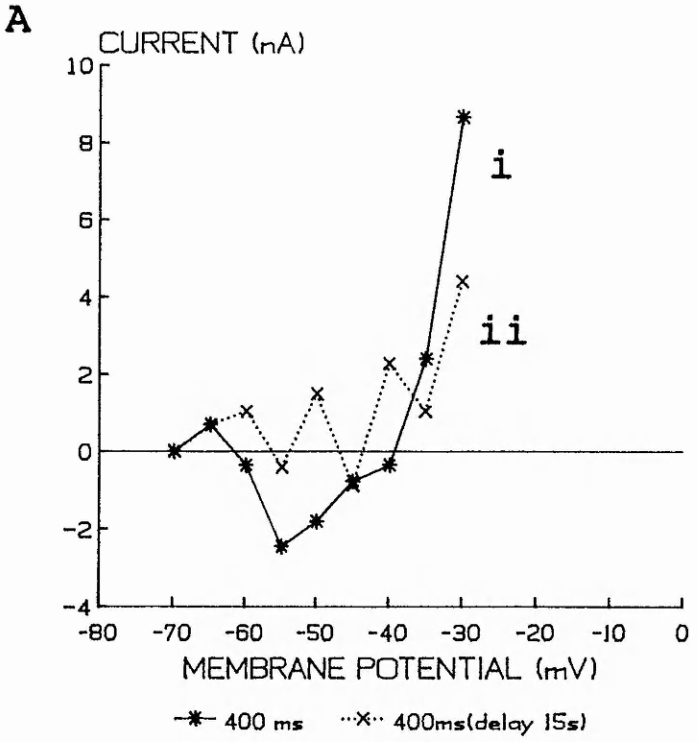
I-V profiles were constructed using the above long-pulse protocol and then the delay between successive pulses shortened to between 10s and 15s for a subsequent I-V run. I-V relationships at 400 and 600 ms for one such neurone, using step delays of 1 minute and 15s, are given in Fig. 3.19. Using the 1 minute step-delay protocol the I-V relationship contains a distinct region of NSR at each time-point which, for this cell, was sufficiently strong to take the relationship below the zero-current (voltage) axis (indicating the development of a net inward current over the potential range of the NSR) (Fig 3.19Ai, Bi). However, when the delay between voltage commands was reduced to 15s the I-V relationship became irregular (Fig 3.19Aii, Bii). Under current-clamp, the post-plateau afterhyperpolarisation lasted for some 50-60 seconds for this cell. It is likely, therefore, that the neurone was not given adequate time to fully recover its resting state between successive voltage steps using the short delay protocol. One explanation for the irregularity of the I-V relationship obtained using the short delay protocol is that inactivation of the inward current carrying the rising phase of the plateau occurs, making the cell refractory to some command pulses: command pulses immediately following a suprathreshold step would be unable to activate

currents mediating plateau potentials, whereas those applied after a suitable delay would again be suprathreshold. This suggests that some minimum time period is required for the current to de-inactivate. Such a delayed recovery from inactivation would cause the irregularities in the I-V relationship shown in Fig. 3.19.

The sensitivity of the delayed inward current component to stimulus frequency is illustrated in Fig. 3.20. This shows the inward component developing in a neurone in response to a command step to -50mV and responses to the same command step at 10s, 15s and one minute, each following after an initial suprathreshold response. With a 10s delay the inward component was entirely absent; with a 15s delay a partial response was obtained. Only with a minute delay was a response similar to the initial current obtained.(The rapid downward deflections superimposed on records 1 and 4 represent breakthrough to the soma of fast events from unclamped portions of the neurone: these most probably correspond to the axon spikes which surmount the plateau under current-clamp.) These observations, under voltage-clamp, are in accord with those made under current-clamp (Fig. 3.5 and Fig. 3.14) and suggest that the refractoriness observed under current clamp results in part from inactivation of channels carrying the inward current of plateau potentials. The recovery from this inactivation will determine the

absolute refractory period between successive plateaux.

FIGURE 3.19: I-V relationships from a neurone at 400ms and 600ms into command pulses (A and B respectively). For each time-point, curve i (solid line) denotes the relationship using a 1 minute delay between successive voltage-clamp pulses, whilst curve ii (dotted line) denotes the relationship when the step-delay was 15 s. The I-V relationship became irregular (particularly in A) when the step-delay was reduced to 15 s. Because of this, successive points are joined with straight lines, rather than the more usual curve-of-best-fit.


A
CURRENT (nA)
10
8
6
4
2
0
-2
-4
i
ii
-80 -70 -60 -50 -40 -30 -20 -10 0
MEMBRANE POTENTIAL (mV)
400 ms
400ms(delay 15s)

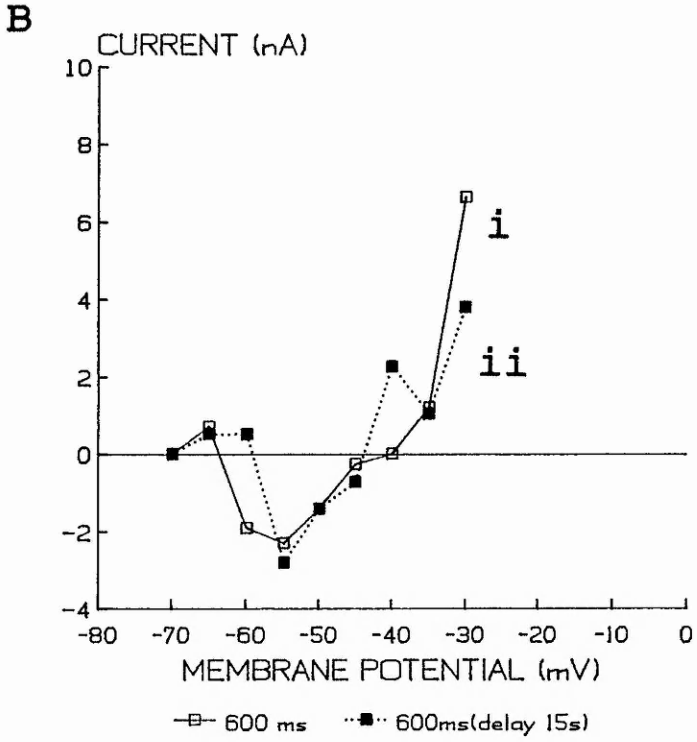
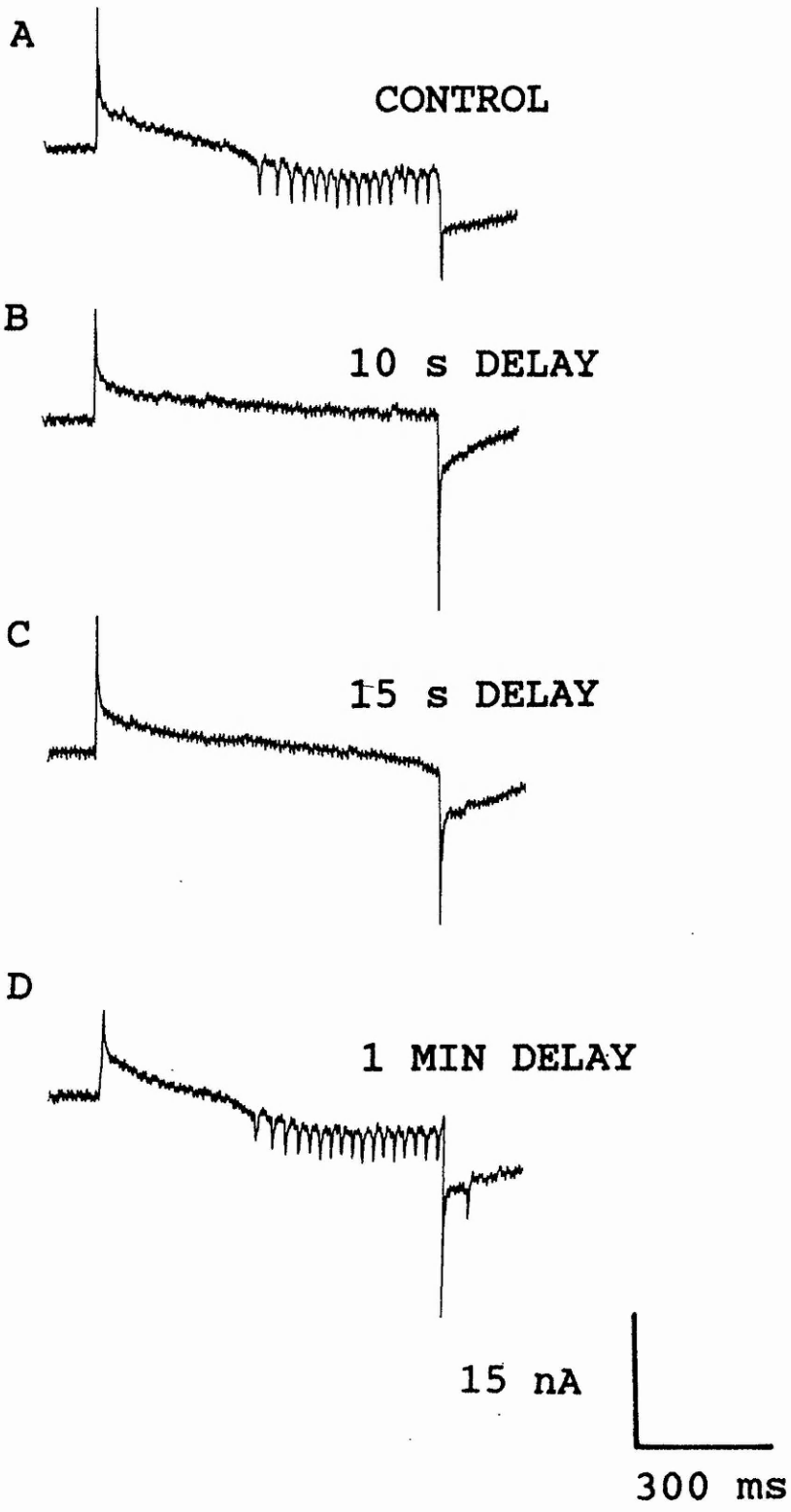

B
CURRENT (nA)
10
8
6
4
2
0
-2
-4
i
ii
-80 -70 -60 -50 -40 -30 -20 -10 0
MEMBRANE POTENTIAL (mV)
600 ms
600ms(delay 15s)

FIGURE 3.20: Net membrane currents (filtered at 45.5 Hz) recorded from a neurone in response to voltage steps from the holding potential (-70mV) to -50mV. Pairs of command pulses were applied; in each case the first pulse elicited a response similar to (A): a slowly developing inward current associated with plateau production. (B) With a pulse-delay of only 10 s no inward current could be evoked by the second pulse . (C) With a 15 s delay between pulses, the second pulse activated some inward current. (D) When the delay between a pair of pulses was increased to 1 minute both voltage commands elicited similar magnitude inward currents.


A
CONTROL
B
10 s DELAY
C
15 s DELAY
D
1 MIN DELAY
15 nA
300 ms

3.3. Ionic dependence of plateau potentials

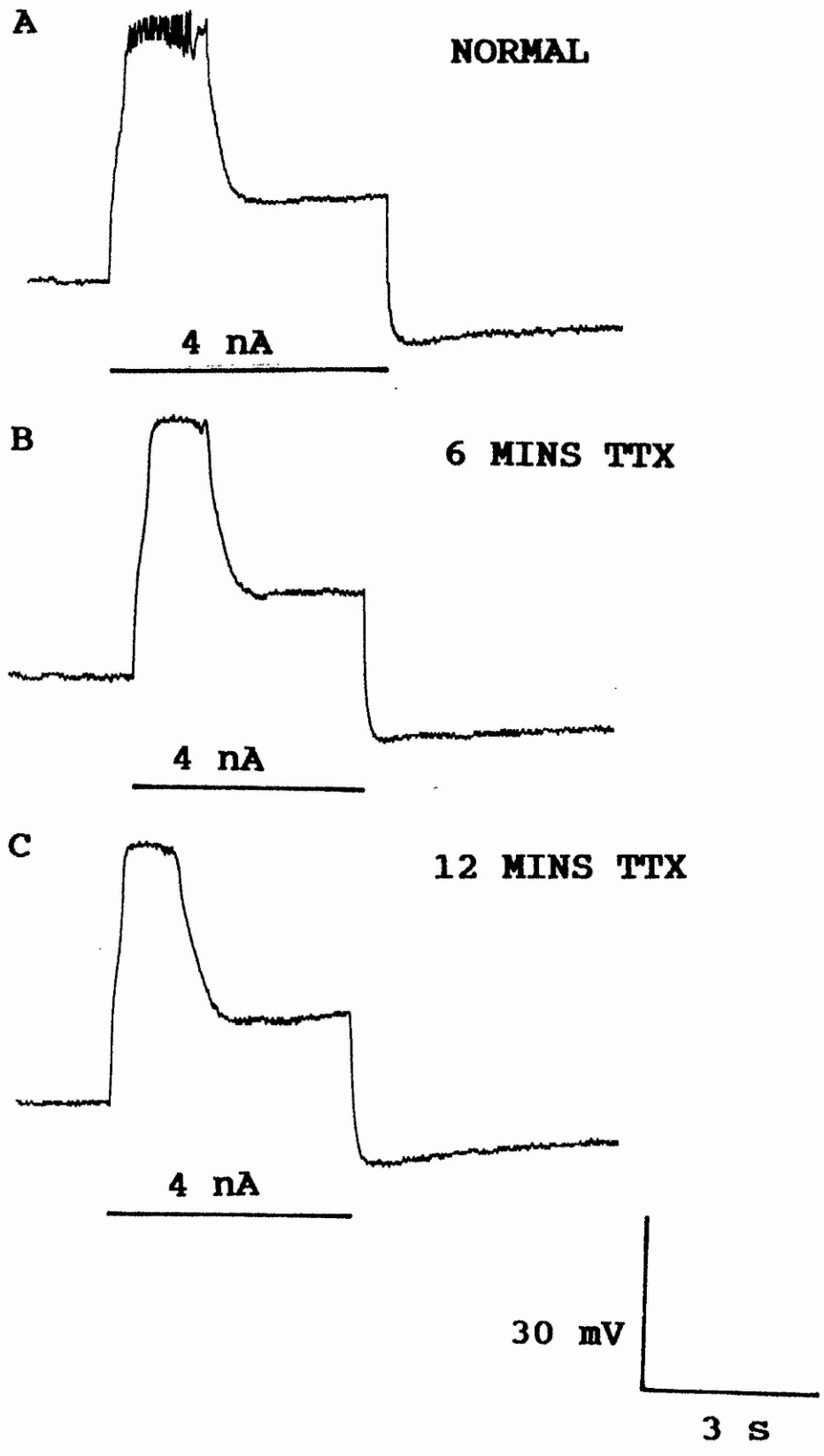
Experiments were performed to establish the ionic basis of plateau potentials.

3.3.1. Intact Neurones

A. Tetrodotoxin

Tetrodotoxin (TTX) has been shown to be effective in blocking sodium-dependent action potentials in D_f. The action potentials which follow anoxia pretreatment have been blocked by concentrations of TTX as low as 2 x 10^{-7}M (Pitman, 1988). TTX was superfused over preparations exhibiting plateau potentials in response to current injection at a concentration of 1 x 10^{-6}M. Plateaux persisted in this concentration of TTX, however the axon spikes surmounting the crest of the plateau response were blocked within several minutes of application. Fig. 3.21 shows plateau potentials recorded prior to TTX application and after six and twelve minutes exposure to TTX. Clearly, then, any sodium-mediated component of the plateaux in this cell is carried by channels which are TTX insensitive and, therefore, distinctly different from the channels which carry the inward current of the axon spikes which surmount the crest of the plateau response.

FIGURE 3.21: Effect of TTX on plateau potential production by D_f. (A) Plateau potential elicited by a depolarising current pulse applied to a cell in normal saline. (B) Plateau potential recorded from same cell 6 minutes after application of 10^{-6}M TTX. Although axon spikes no longer surmount the crest of the plateau response, the plateau potential itself appeared to be little affected by TTX. (C) Plateau potential recorded from same cell 12 minutes after application of TTX. Cell resting potential -78mV. Horizontal bars represent duration of applied current pulses.


A
NORMAL
4 nA
B
6 MINS TTX
4 nA
C
12 MINS TTX
4 nA
30 mV
3 s

B. Cadmium chloride and manganous chloride

Plateaux were sensitive to both cadmium chloride (1-2 mM) and manganous chloride (40mM/20mM) (n=8). Both agents blocked plateau potentials in the intact neurone. Fig. 3.22 shows the effects of $CdCl_2$ on plateaux. Following blockade, substantial increases in injected current were unable to evoke plateaux. Blockade was often preceded by a progressive depolarisation of the cell, during which spontaneous plateaux could be observed. This could be explained in terms of effects of these agents on the calcium dependent potassium current (I_C) present in this neurone (Thomas, 1984); this conductance appears to play a role in determining the resting potential of D_f (David and Sattelle, 1990). Under voltage-clamp, application of these agents abolished the NSR in the I-V profile of cells exhibiting plateaux. Fig. 3.23 shows the effect of 20mM $MnCl_2$ on the I-V relationship. Interestingly, the I-V profile in $MnCl_2$ intersects that in the untreated cell positive to -40mV, indicating that a proportion of the outward rectifying current is calcium-dependent. This observation is consistent with the effects of zero-Ca^{2+} saline on the I-V relationship for D_f and the effects of Cd^{2+} ions on the I-V relationship of cell 3 (Thomas, 1984; Nightingale and Pitman, 1989).

In intact cells, the effects of these agents were often relatively slow in onset (up to 20 minutes) and

difficult to reverse. One possible explanation for this is that the regional distribution within the neurone of calcium channels involved in plateaux is such that, in the intact preparation, access by Cd^{2+} and Mn^{2+} ions was difficult.

FIGURE 3.22: Effect of Cd^{2+} ions on plateau potential production by D_f. (A) Plateau potential series recorded from a cell in response to a long duration depolarising current pulse. (B) The same magnitude pulse failed to elicit a plateau 2 minutes after applying Cd^{2+} ions (1mM). (C) A twofold increase in injected current similarly failed to elicit a plateau, although the cell did not respond entirely passively. Cell resting potential -70mV. Horizontal bars represent duration of applied current pulses.

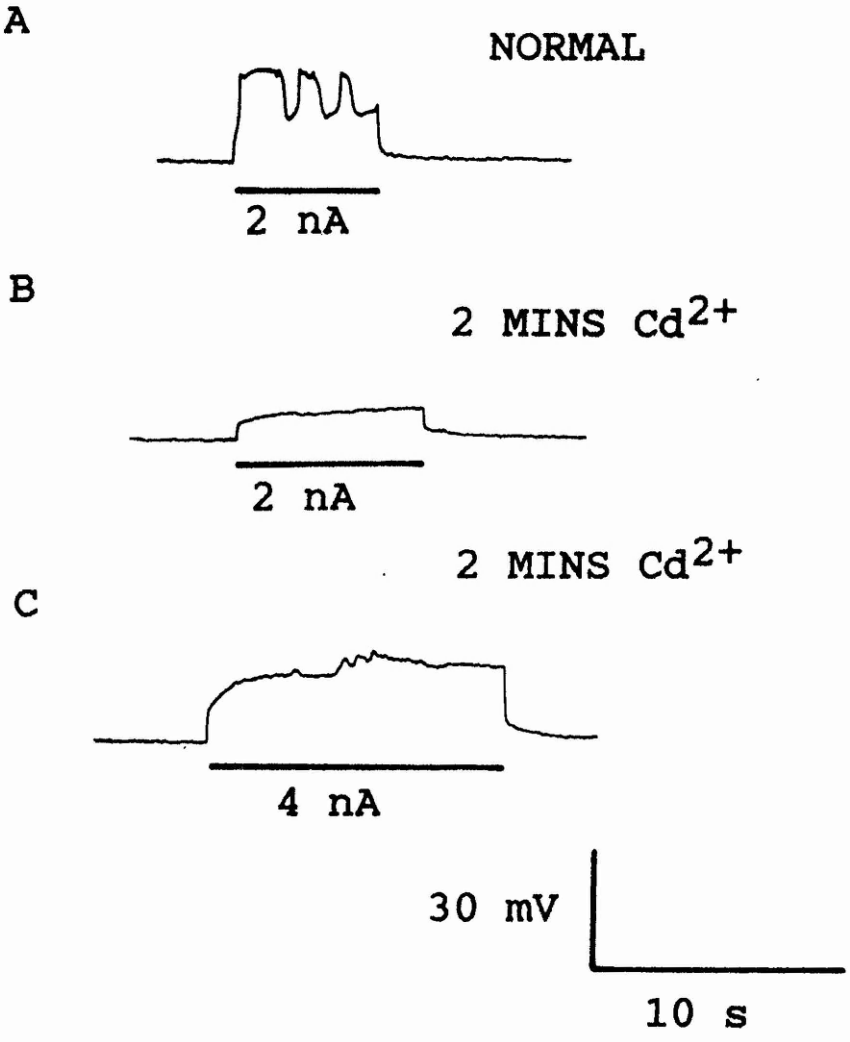
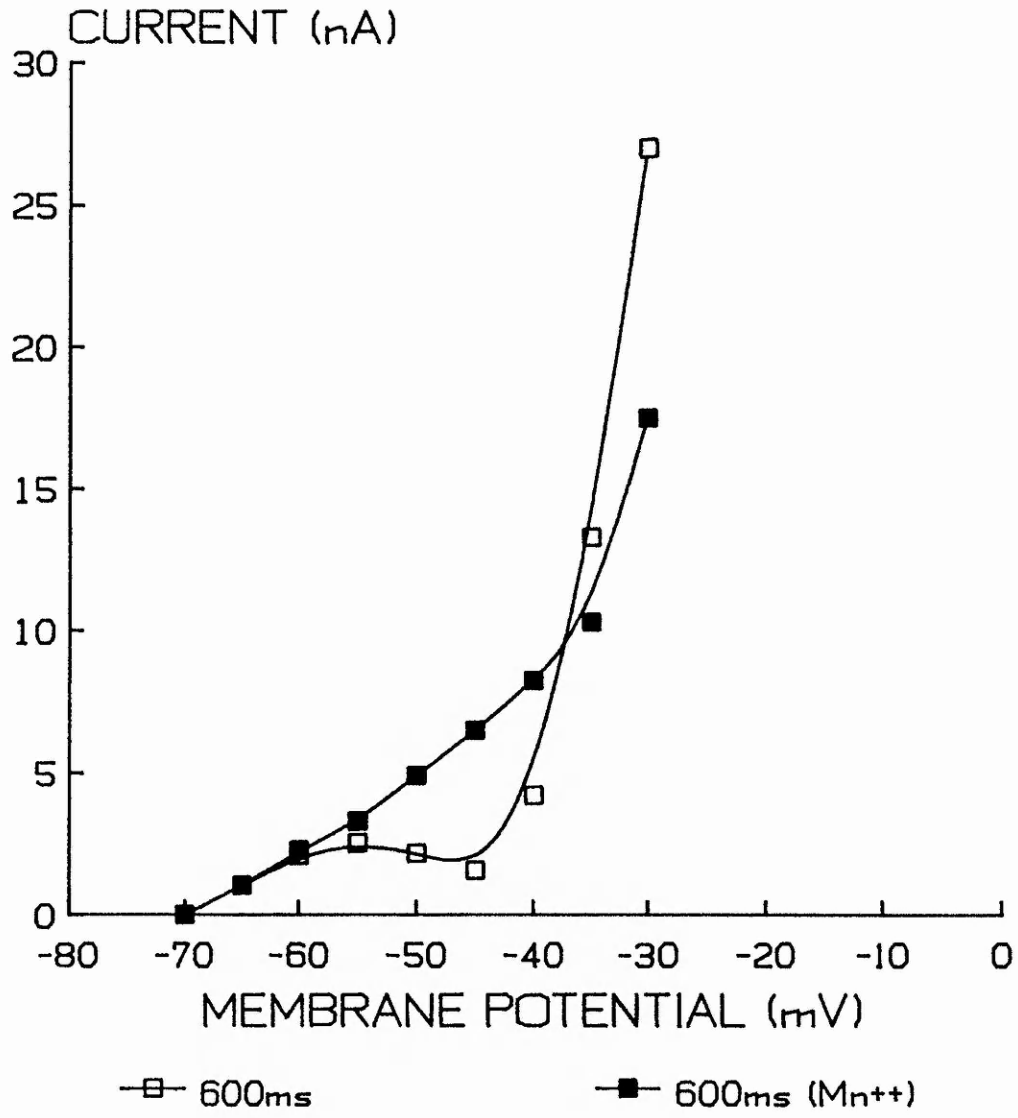

A
NORMAL
2 nA
B
2 MINS Cd2+
2 nA
2 MINS Cd2+
C
4 nA
30 mV
10 s

FIGURE 3.23: Current-voltage relationships for a neurone exhibiting plateau potentials before (open squares) and 10 minutes after (filled squares) application of 20mM Mn^{2+} ions. Mn^{2+} ions eliminated the region of NSR in the I-V relationship. I-V curves constructed from data sampled 600ms into current responses. Holding potential -70mV.


CURRENT (nA)
30
25
20
15
10
5
0
-80
-70
-60
-50
-40
-30
-20
-10
0
MEMBRANE POTENTIAL (mV)
600ms
600ms (Mn++)

3.3.2. Mechanically isolated somata

To limit the effects of access problems, further investigations of the ionic dependence of plateaux were carried out using mechanically isolated somata (n=8).

A. Low sodium saline

To look at the sodium-dependence of somatic plateaux, cells were exposed to low-sodium rather than sodium-free saline. This is because it was judged that complete removal of sodium ions from the external environment could have potentially serious implications in terms of data interpretation as a result of indirect effects of sodium removal on any Na/Ca exchange mechanism in the soma membrane. A number of experiments (n=3) using sodium-free saline appeared to confirm this rationale: in these experiments cells hyperpolarised following addition of sodium-free saline and their input resistances decreased markedly; both observations are consistent with intracellular calcium accumulation and a resultant increase in I_C. A further justification for using low sodium saline is that sodium removal could affect intracellular pH, since there is evidence that insect neurones utilise a Na^+/H^+ exchanger in intracellular pH regulation (Schwiening and Thomas, 1989).

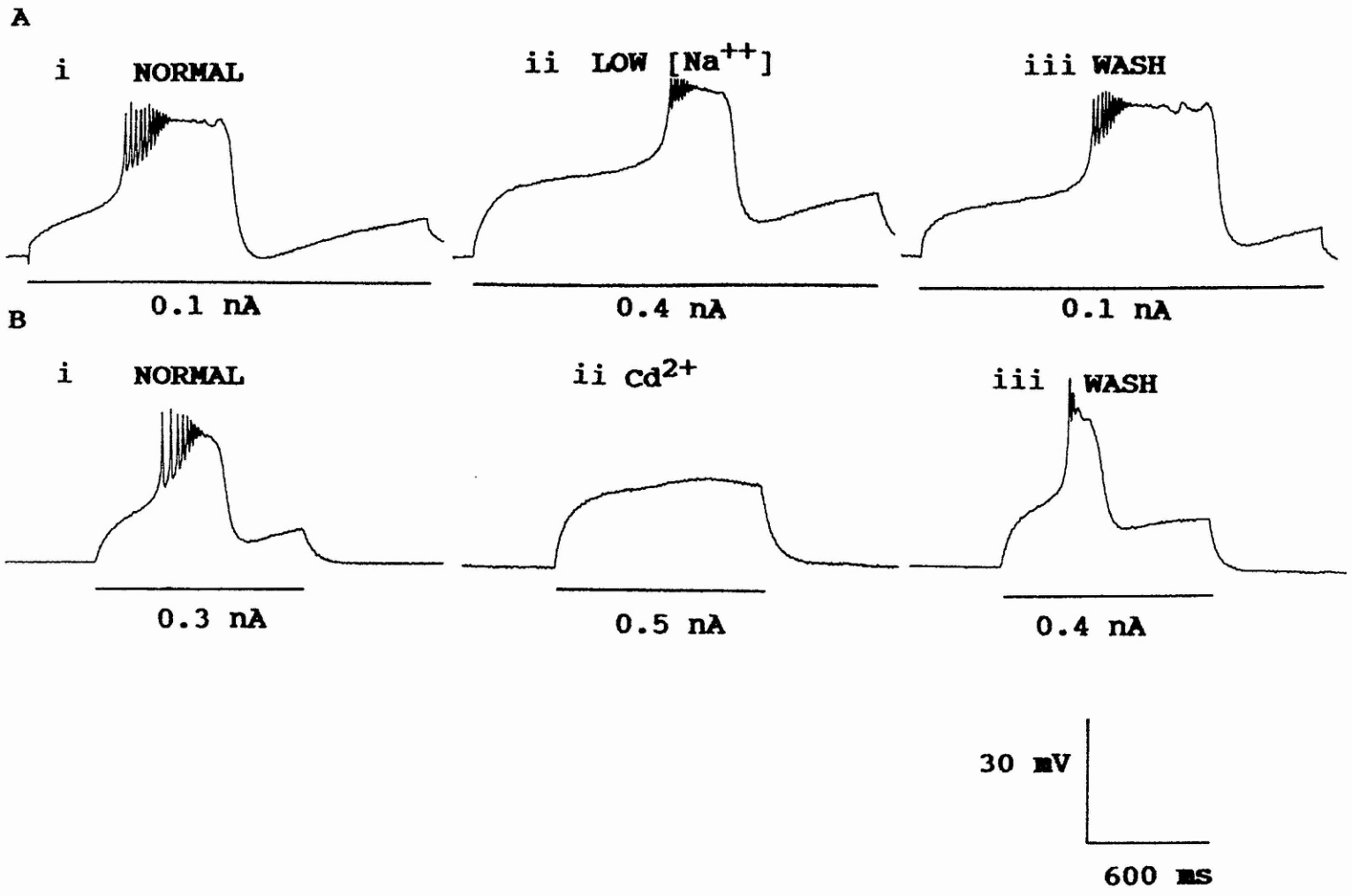
For the above reasons, then, cells generating plateaux were superfused with saline containing a quarter of the normal sodium concentration (see APPENDIX I). Plateau potentials persisted in low-sodium saline, although their durations were shorter than those recorded in standard saline (Fig 3.24A). Cells hyperpolarised by between 5-10mV when the perfusate was changed to low-sodium saline; this raised the threshold current for plateaux. On return to standard saline cells recovered their resting potentials. Presumably, the effect on the cell resting potential could be interpreted, to a degree, in terms of changes in intracellular Ca consequent upon changes in [Na] of the superfusate.

Plateaux from mechanically isolated somata were also resistant to bath applied TTX at concentrations of up to 10^{-5}M (not shown).

B. Cadmium chloride

Plateau potentials were completely and reversibly abolished in the presence of 1mM $CdCl_2$ (Fig 3.24B). This indicates that the inward current underlying the plateau potentials recorded from isolated somata is predominantly carried by calcium ions.

FIGURE 3.24: Ionic dependence of plateau potentials from somata mechanically isolated *in situ*. (A) Effect of low sodium saline. Reduction of the external sodium concentration to one quarter of normal (from 214mM to 54mM) shortened but did not abolish plateaux. (B) Application of 1mM Cd^{2+} ions to a different preparation rapidly (within 6 minutes) abolished plateaux. This effect was reversible. Resting potentials (A) -65mV; (B) -70mV. Horizontal bars represent duration of applied current pulses.


A
i NORMAL
ii LOW [Na++]
iii WASH
0.1 nA
0.4 nA
0.1 nA
B
i NORMAL
ii Cd2+
iii WASH
0.3 nA
0.5 nA
0.4 nA
30 mV
600 ms

3.3.3. Ca channels involved in plateau generation differ from those mediating action potentials

The preceding experiments demonstrated that a significant proportion, if not all, of the inward current mediating plateaux in D_f appears to be calcium-dependent. An obvious question arising from these experiments was "Are the calcium channels carrying inward current during plateaux distinct from those which carry the inward current of calcium-dependent action potentials following pharmacological treatment of the neurone; or are plateau potentials a symptom of changes to fast calcium channels in the soma membrane?"

Eight cells were injected with citrate anion (see chapter 2; section 2.2.6) and their responses to short and long pulse protocols recorded under current-clamp. I-V profiles were constructed for seven of these neurones under voltage-clamp.

Following 5-10 minutes of citrate injection, each of these cells responded to relatively short duration (50 to 200ms), suprathreshold pulses with all-or-none action potentials, the number of which increased with the magnitude and duration of the applied current (Fig. 3.25).

In four of the eight experiments, current pulses of considerably longer duration (up to several

seconds) elicited plateau potentials (Fig 3.26). All-or-none action potentials were visible on the rising phase of these plateaux (Fig 3.26B_i , $B_{i\,i}$) and in some instances the action potentials surmounted the crest of the plateau response (Fig. 3.26C). These action potentials closely resembled those evoked by the shorter duration pulses following citrate injection, differing greatly from the axon spikes which often surmount the crest of the plateau response in the untreated neurone (this difference is highlighted in Fig. 3.6). The peak-trough amplitude of spikes on the rising phase of plateaux decreased towards the crest of the response, the after-spike hyperpolarisations decrementing more than the peak amplitudes of the action potentials. Presumably this progressive decrement resulted in part from conductance changes during the plateau.

These findings strongly indicated that two different calcium-dependent events could occur simultaneously in the same neurone and that, since plateaux and citrate-spikes could co-exist, these two events would necessarily be mediated by different classes of calcium channel.

FIGURE 3.25: (A) Response of a neurone to a subthreshold depolarising current pulse after ten minutes of intracellular citrate injection. (B) All-or-none action potential elicited by a larger current pulse. (C) A sufficiently large depolarisation could elicit a series of action potentials, each with its own distinct afterhyperpolarisation. Cell resting potential -72mV. Horizontal bars represent duration of applied current pulses.

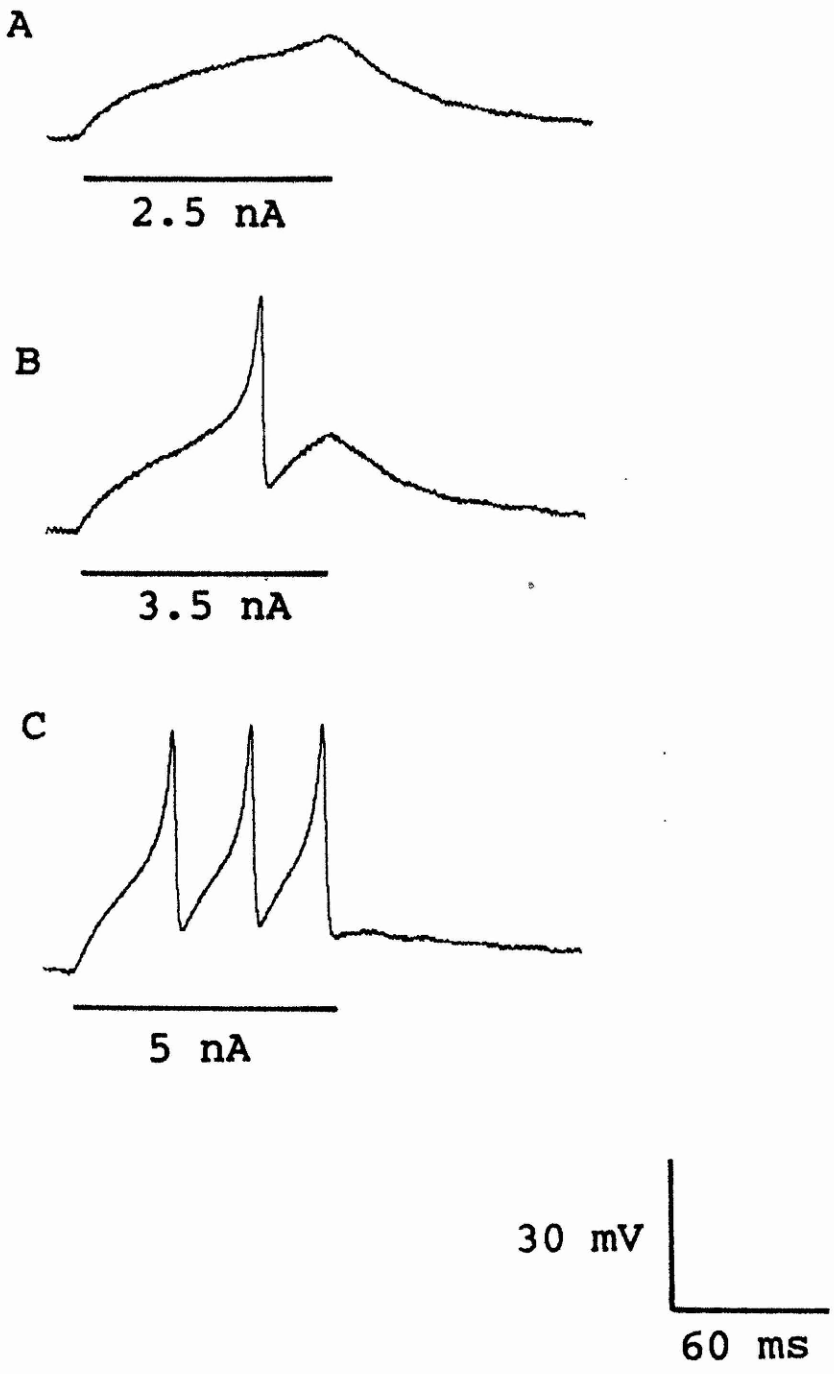
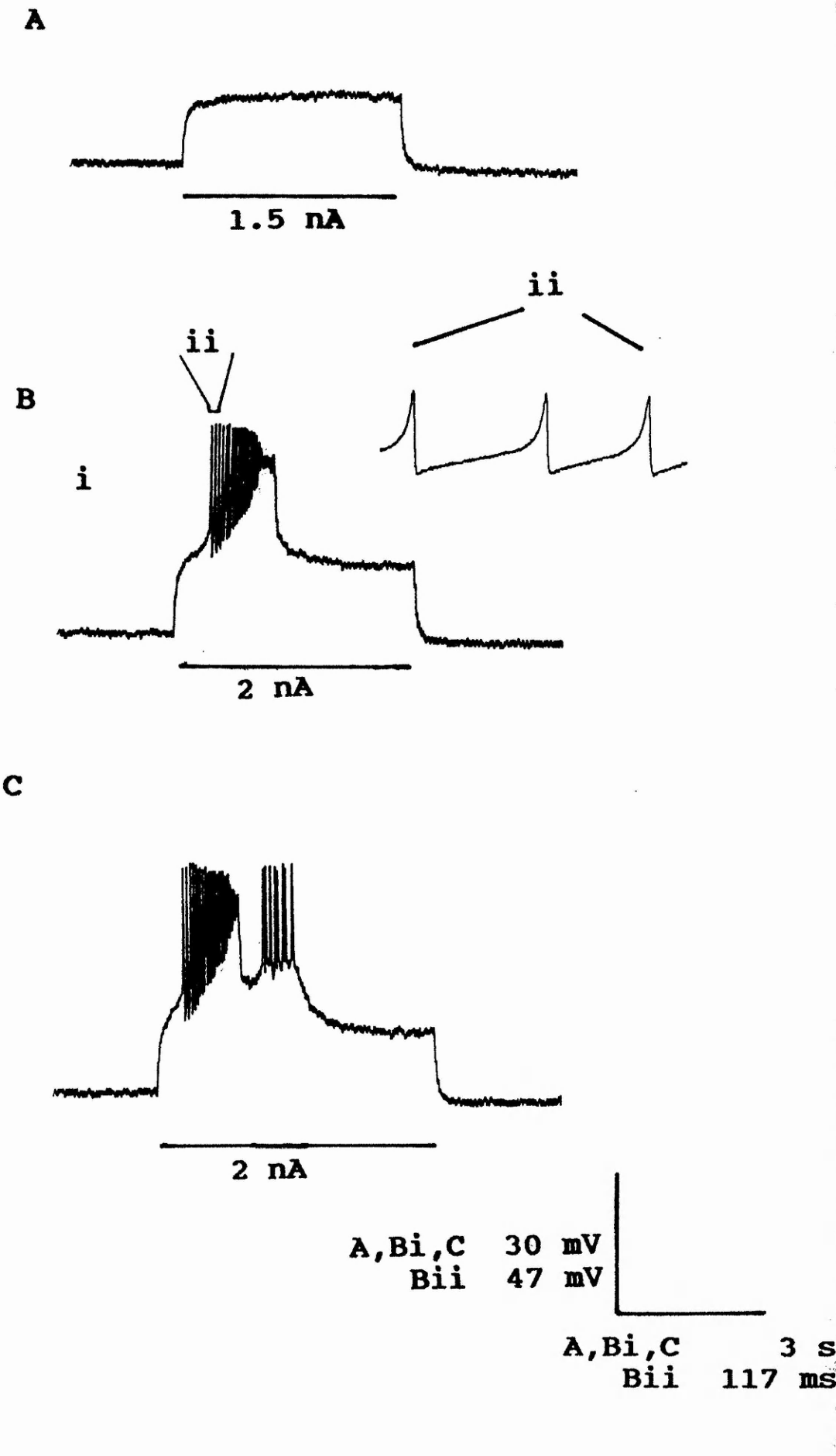

A
2.5 nA
B
3.5 nA
C
5 nA
30 mV
60 ms

FIGURE 3.26: Response of same neurone as that in Fig. 3.25. to much longer duration current pulses. Cell had been injected with citrate anion for ten minutes. (A) Response to a subthreshold depolarisation. (Bi) Response to a suprathreshold depolarisation: cell generated a plateau potential, on the rising phase of which were citrate-induced action potentials. This is shown more clearly in (Bii) which shows the first three action potentials of the series on an enlarged time-scale. (C) Response of the same cell to a subsequent depolarisation: citrate-induced action potentials can surmount the crest of a plateau. Cell resting potential -72mV. Horizontal bars represent duration of applied depolarisation.


A
1.5 nA
ii
ii
B
i
2 nA
C
2 nA
A,Bi,C 30 mV
Bii 47 mV
A,Bi,C 3 s
Bii 117 ms

Co-existence of plateau potentials and citrate-induced action potentials was further investigated under voltage-clamp. Fig. 3.27 shows selected current responses from a voltage-clamp experiment performed on a neurone exhibiting citrate-induced action potentials, but not plateau potentials. Current components early in the responses to depolarising command steps were different to those from non-plateauxing neurones in which citrate had not been injected. Positive to between -50mV to -40mV (this varied between preparations) inward currents began to develop significantly in the early part of the current response. These relatively early inward currents increased in magnitude up to -30mV to -20mV, positive to which they were masked by the development of strong outward currents. Inward currents were rapid in their activation, rapidly reaching their peak values for a given voltage step. However, these inward currents were not sustained throughout the duration of the applied step; the later currents were predominantly outwardly directed.

I-V profiles from such cells were of the form given in 3.28A. The profile constructed from data sampled 50ms into responses showed a large region of negative slope resistance between -50/-40mV and -30/-20mV which for all but one neurone transected the voltage axis. For such cells, I-V profiles constructed from data sampled at 600ms showed much smaller regions of NSR in this same voltage range.

The negative resistance region in the 'early' I-V profile from neurones after citrate injection was sensitive to Mn^{2+} ions (Fig.3.28B) indicating that the inward current underlying this region is calcium-dependent. This correlates with the previous observation made under current-clamp that citrate-induced action potentials are calcium-dependent (Pitman, 1979).

FIGURE 3.27: Sample currents (filtered at 111Hz) recorded under voltage-clamp from a neurone generating citrate-spikes but not plateau potentials. Holding potential -70mV. Arrows indicate points at which currents were sampled to construct I-V relationships for Fig. 3.28A.

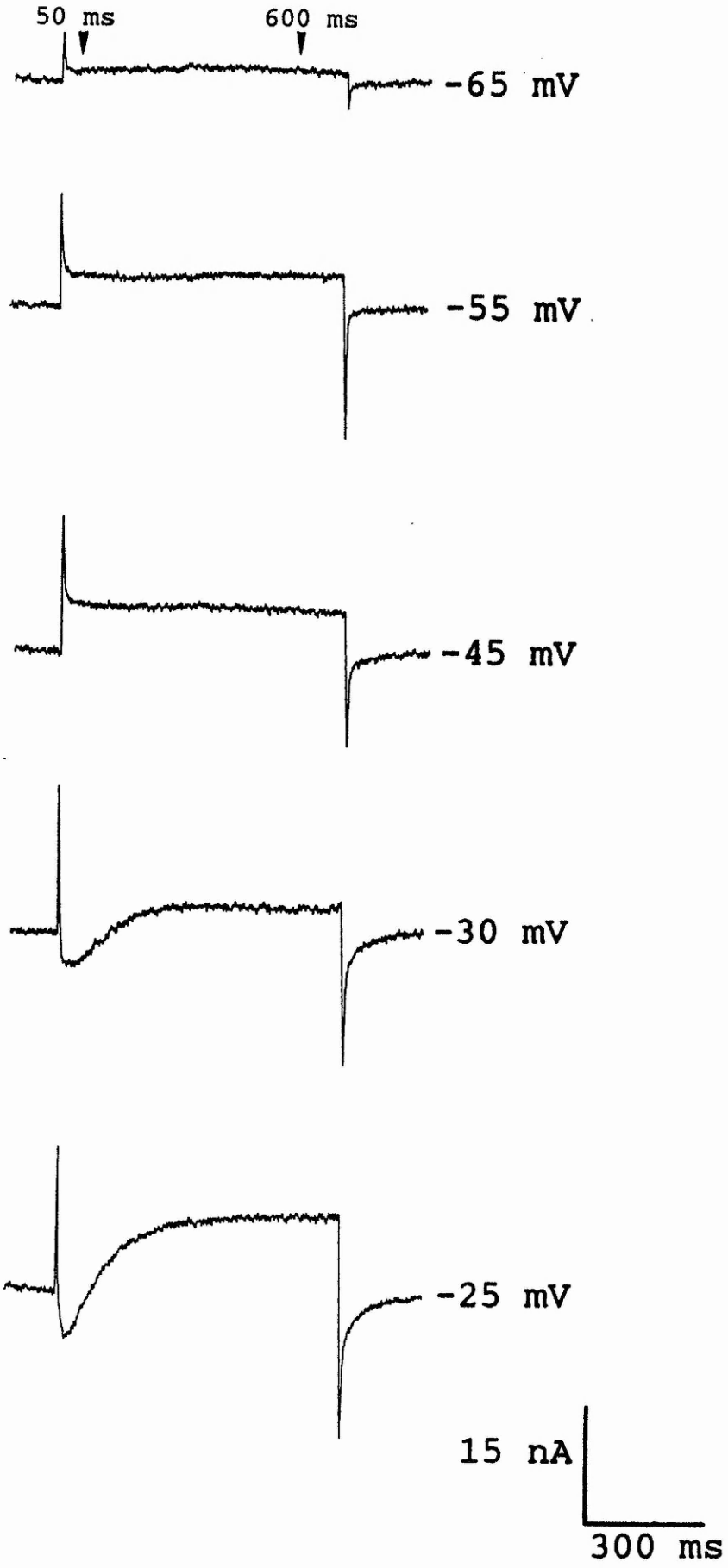
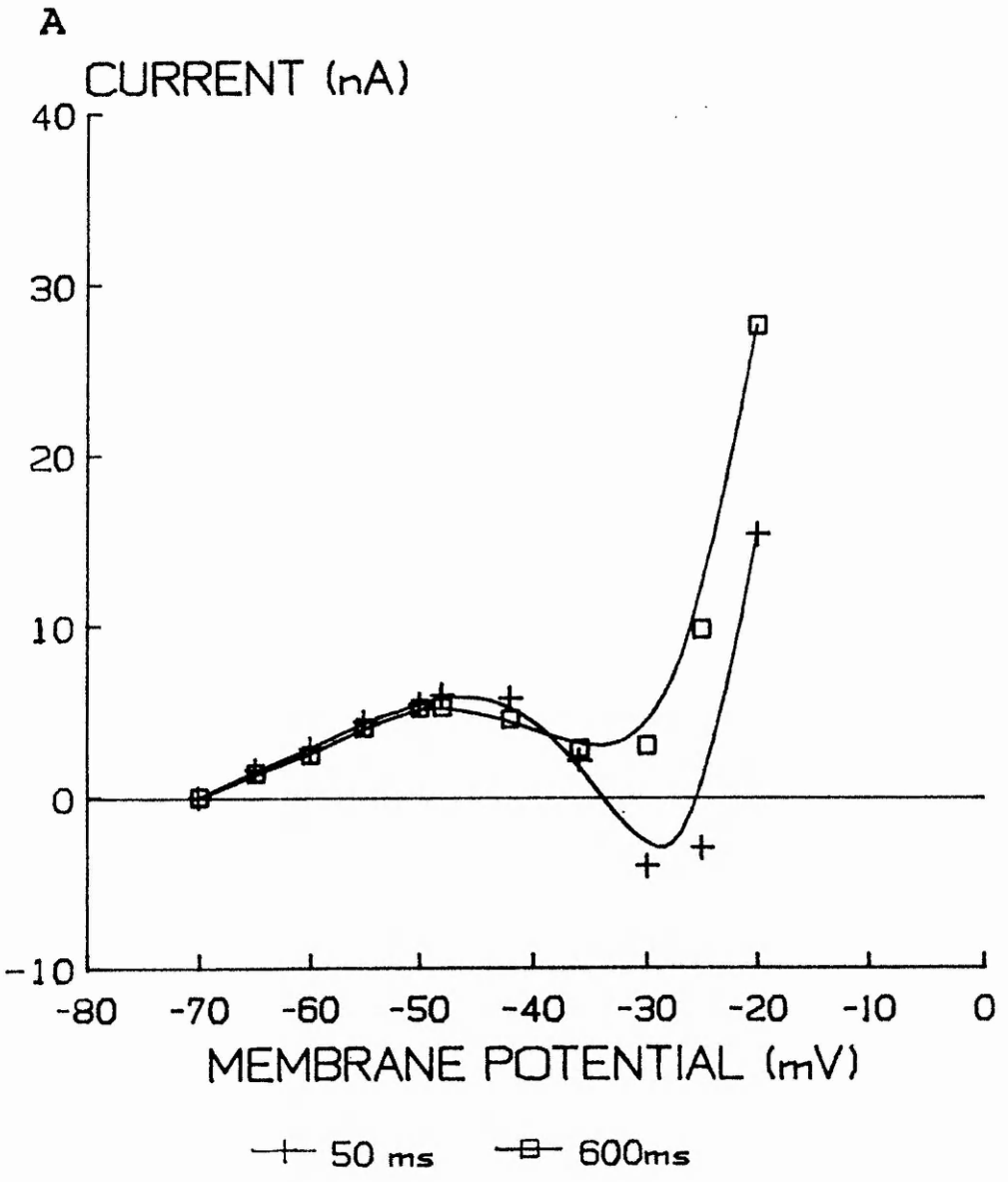
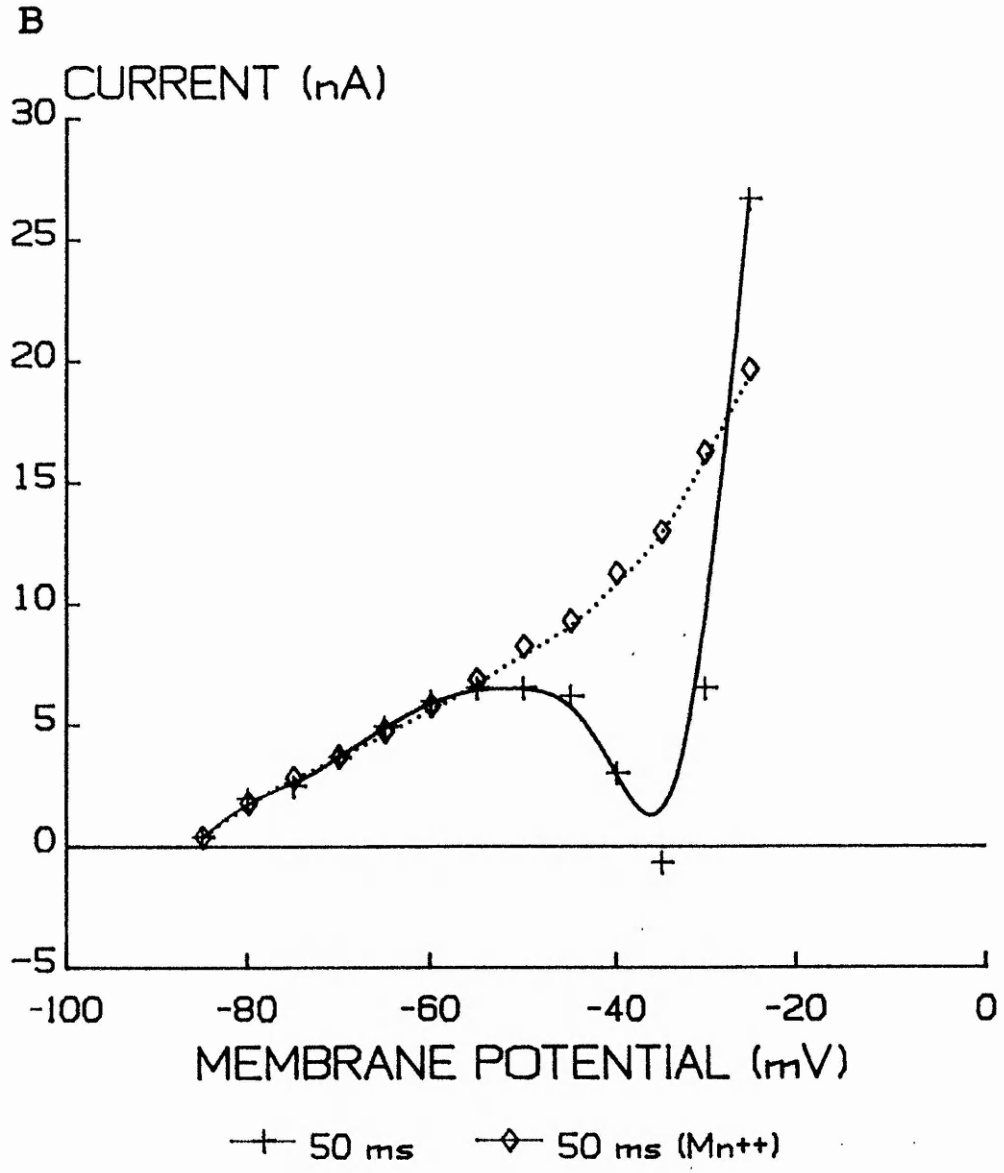

50 ms
600 ms
-65 mV
-55 mV
-45 mV
-30 mV
-25 mV
15 nA
300 ms

FIGURE 3.28: (A) (opposite) Current-voltage relationships at 50ms (crosses) and 600ms (open squares) into currents sampled under voltage-clamp for a neurone generating citrate-spikes but not plateau potentials (same cell as used for Fig. 3.27).

(B) (overleaf) Effect of Mn^{2+} ions (20mM) on the I-V relationship of a different cell injected with citrate ions (15 minutes citrate injection). Currents were sampled at 50ms into current responses before (crosses) and ten minutes after (open diamonds) addition of Mn^{2+} ions. Mn^{2+} ions abolished the region of negative resistance associated with citrate-spike production. Holding potential -85mV.


A
CURRENT (nA)
40
30
20
10
0
-10
-80
-70
-60
-50
-40
-30
-20
-10
0
MEMBRANE POTENTIAL (mV)
50 ms
600ms


B
CURRENT (nA)
30
25
20
15
10
5
0
-5
-100
-80
-60
-40
-20
0
MEMBRANE POTENTIAL (mV)
50 ms
50 ms (Mn++)

Voltage-clamp experiments performed upon individual neurones exhibiting both citrate-induced action potentials and plateau potentials provided substantial evidence indicating that the two events involve different classes of calcium-channel.

Current records from one such experiment are shown in Fig. 3.29. Positive to -60mV a slowly activated inward current became superimposed on the net outward current response. This relatively slow inward current increased until -40mV, the activation being moved earlier into the response at more positive potentials in this range. Superimposed on each current response positive to -45mV are series of downward deflections. These represent breakthrough to the soma of short-duration, active events from unclamped portions of the neurone. In fact, these deflections most probably resulted from the electrotonic conduction of axon spikes into the soma at potentials above the threshold for plateau potentials. The slow inward current apparently corresponded to that identified for untreated neurones generating plateaux, and was not a result of citrate injection since it was absent from citrate-treated neurones in which plateaux were not observed.

The early current component in this neurone also exhibited a voltage-dependent inward current. This component was activated at more positive potentials than the slowly developing inward current; it appeared not to develop until -40mV in this cell, at which

potential the slow component was near-maximal. This early component correlated with that in citrate injected cells in which plateaux had not been observed.

I-V profiles for this cell are given in Fig. 3.30. The I-V relationship at 50ms is similar to that at the same timepoint in Fig 3.28; the trough lies at -30mV in both neurones. At 600ms however, the I-V relationship demonstrates an area of NSR in a more negative voltage range (-55mV t0 -35mV) which is absent at the corresponding time in the I-V relationships of citrate-injected neurones that did not exhibit plateau potentials.

Thus, in cells exhibiting fast, calcium-dependent action potentials and slow plateau potentials with a strong calcium-dependence, two discrete regions of NSR with discrete voltage dependencies were identified in relatively early and late I-V relationships respectively. Under current clamp, plateaux and citrate-induced spikes could be recorded simultaneously. Therefore, at least two populations of calcium channels exist in D_f, capable of mediating fast and slow events. This finding is important: it demonstrates that plateaux are not an artefact resulting from changes to the channels mediating fast events in this cell, rather they are discrete events mediated, at least in part, by previously unidentified calcium channels.

FIGURE 3.29: Sample currents (filtered at 111Hz) recorded under voltage-clamp from a neurone generating citrate-spikes and plateau potentials. Holding potential -65mV. Arrows indicate points at which currents were sampled to construct I-V relationships for Fig. 3.30.

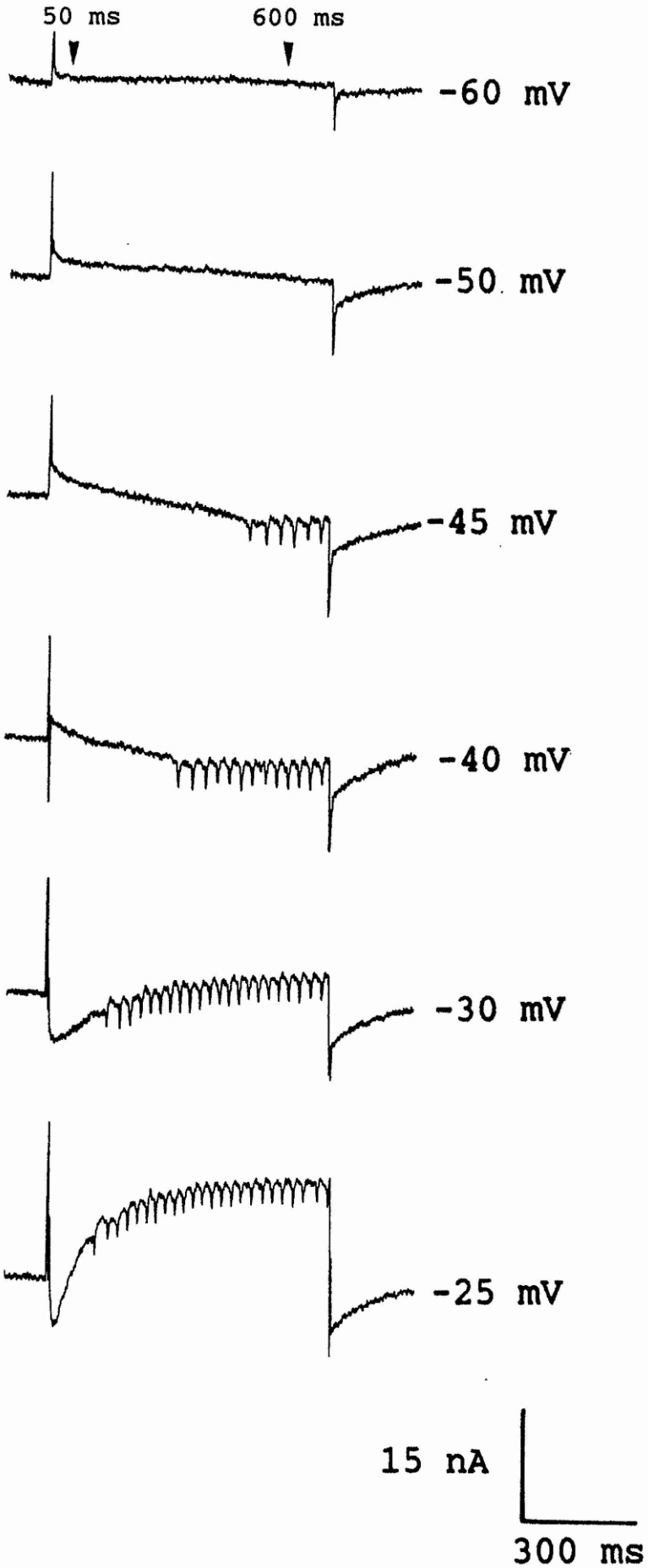
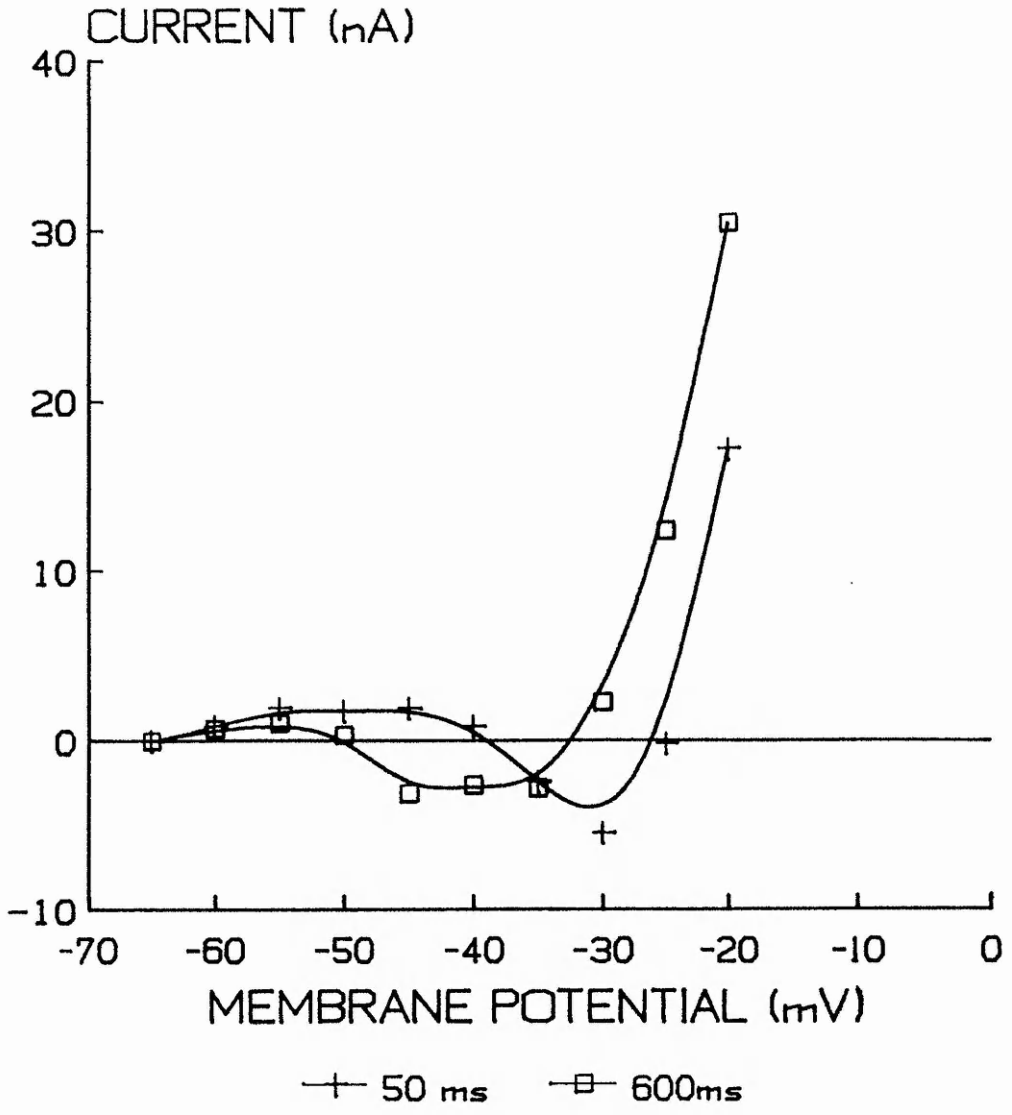

50 ms
600 ms
-60 mV
-50 mV
-45 mV
-40 mV
-30 mV
-25 mV
15 nA
300 ms

FIGURE 3.30: Current-voltage relationships at 50ms (crosses) and 600ms (open squares) into currents sampled under voltage-clamp for a neurone generating citrate-spikes and plateau potentials (same cell as used for Fig. 3.29).


CURRENT (nA)
40
30
20
10
0
-10
-70
-60
-50
-40
-30
-20
-10
0
MEMBRANE POTENTIAL (mV)
50 ms
600ms

3.4. Manipulation of plateau potential production

3.4.1. Gamma-aminobutyric acid (GABA)

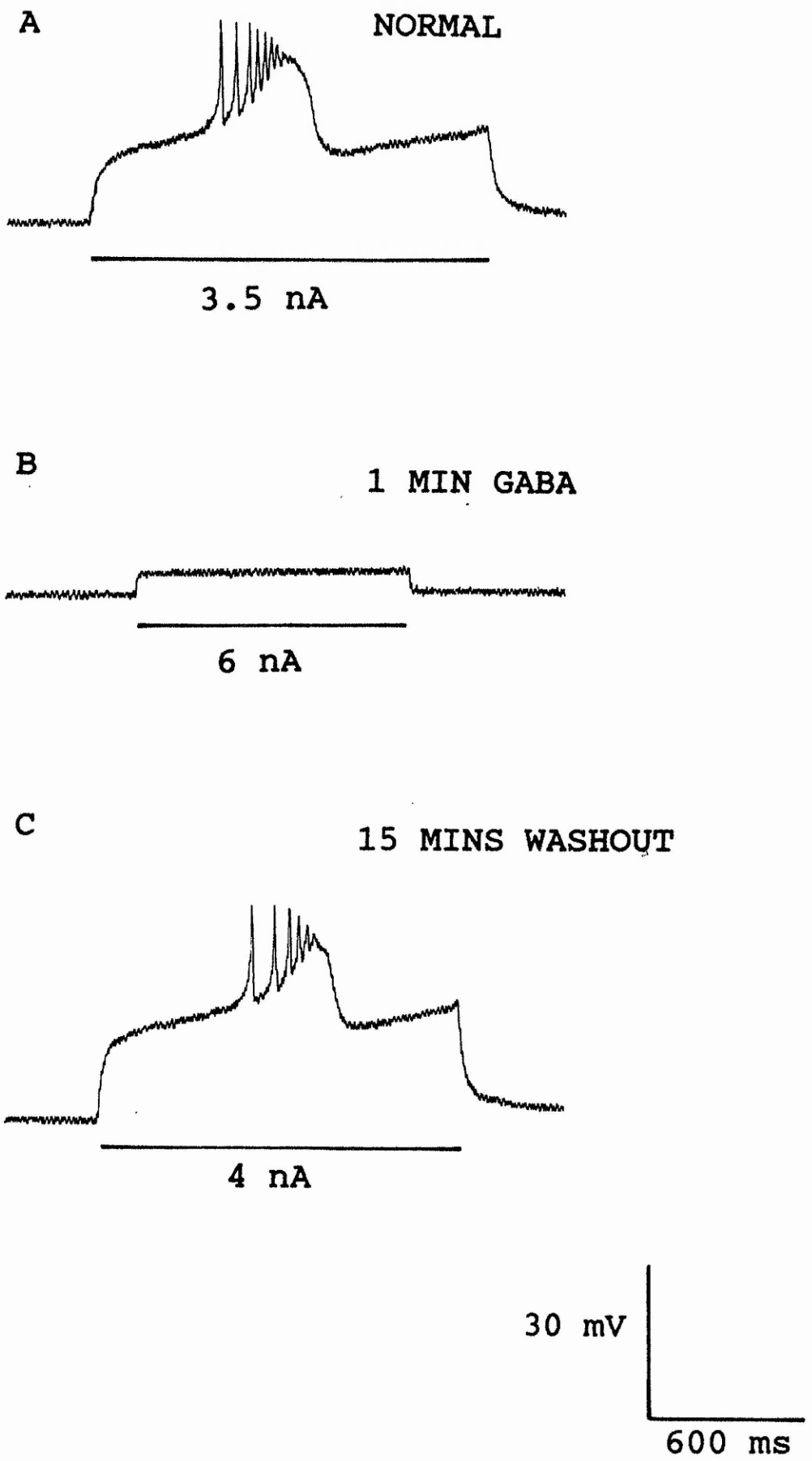
Preparations were superfused with saline containing GABA at concentrations of 10^{-3} to 10^{-4} M (n=5). Shortly after GABA addition (15s to 1 minute) cells were observed to hyperpolarise. They remained hyperpolarised for variable time periods up to three minutes in the presence of GABA, after which they began to repolarise. The effects of GABA on plateau potential production are illustrated in Fig 3.31. In normal saline plateau potentials could be induced by depolarising current pulse injection (Fig. 3.31A). In less than a minute after addition of 10^{-4}M GABA depolarising current pulses were no longer able to produce plateau potentials. This effect was not simply attributable to cell hyperpolarisation, since substantial increases in injected current failed to produce plateaux (Fig. 3.31B). Plateau-abolition in GABA-containing saline was associated with substantial increases in membrane conductance; the input resistance of the cell used for Fig. 3.31 fell from 5MΩ in normal saline to 2MΩ in GABA-containing saline. Following perfusion with normal saline the effects of GABA were reversed (Fig. 3.31C); reversal of the effects of GABA was associated with an increase in input resistance towards levels obtained in the

untreated cell (in this instance the input resistance increased from 2MΩ to 4MΩ on washout).

The qualitative effects of GABA were the same in all preparations; cells hyperpolarised and membrane conductance increased. In all cells 10^{-4}M GABA raised the threshold current for plateaux, and in three eliminated them entirely. In the remaining two, increasing the GABA concentration to 10^{-3}M rapidly abolished plateau potentials. The concentration of the transmitter in the superfusate did not necessarily reflect that at the surface of the neurone; access was variable, probably depending upon how effectively surrounding glial cells had been removed during the desheathing process.

These experiments indicated that at least one endogenous neurotransmitter is able to affect the ability of D_f to generate plateau potentials. Preliminary experiments superfusing the preparation with dopamine, glutamate, proctolin and the muscarinic agonist McN A343 yielded rather variable results.

FIGURE 3.31: Effect of 10^{-4}M gamma-aminobutyric acid (GABA) on plateau potentials from D_f. (A) Plateau potential recorded in normal saline. (B) Response of cell to a larger amplitude depolarisation applied 1 minute after GABA addition. (C) Plateau potential recorded after fifteen minutes washout of GABA. Membrane potentials at which responses were recorded (A) -75mV; (B) -85 mV; (C) -78mV. Horizontal bars represent durations of applied current pulses.


A
NORMAL
3.5 nA
B
1 MIN GABA
6 nA
C
15 MINS WASHOUT
4 nA
30 mV
600 ms

3.4.2. Picrotoxin (PTX)

In eight experiments the GABA antagonist picrotoxin (PTX) was applied to preparations (final bath concentration 10^{-5} M) and its effect on the spontaneous activity of D_f determined.

As described earlier (section 3.1.1), in the absence of applied current the 'normal' D_f can be quiescent, or small excitatory or inhibitory post-synaptic potentials can be seen superimposed on the resting membrane potential. In six of eight preparations examined in this series of experiments, spontaneous plateau potentials were recorded following picrotoxin addition. These events did not appear to be associated with consistent changes in the resting membrane potential of the neurone and only developed with a significant delay; cells first exhibited enhanced fluctuations in membrane potential. Fig. 3.32A$_{i-iv}$ show the effects of PTX on one such preparation. After thirty minutes of PTX exposure, the cell produced spontaneous membrane potential fluctuations which were devoid of a plateau phase (Fig. 3.32A$_{ii}$). At 41 and 45 minutes after PTX addition, the cell generated spontaneous plateau potentials of the type seen in Fig. 3.32A$_{iii}$ and $_{iv}$ respectively (N.B. the difference in timescale between 3.32A$_{iii}$ and $_{iv}$.). In Fig 3.32A$_{iv}$ attenuated action potentials surmounting the crest of the plateau response are visible; these appear similar to those

evoked by plateau potentials in the untreated D_f. In each experiment, this action of PTX was long lasting and, apparently, irreversible: in no instance did the electrical activity of the neurone return to that observed prior to PTX-treatment when preparations were washed in normal saline.

PTX-induced spontaneous plateau-potentials recorded from a second cell, over a 3-4 minute period two hours post PTX treatment, are shown in Fig 3.32B. The discharge pattern of the spontaneous plateaux (the larger amplitude events in this record) was irregular, suggesting that the trigger for plateau generation in PTX-containing saline was synaptic, rather than the result of activation of a slow depolarising current following PTX treatment. In between the larger, plateau-type responses which were of similar amplitude, smaller responses of varying amplitude could be observed. These presumably reflect excitatory inputs subthreshold for plateau production. Individual plateau events were not preceded by slow depolarisations of the membrane potential towards a threshold value, rather they appeared to be switched on rapidly (this also implicates a synaptic trigger). This is shown more clearly in Fig 3.32C which shows spontaneous events in the presence of PTX with zero current injection, and with +2nA and -2nA current injection respectively; in each instance the events take off rapidly from a 'steady' resting potential.

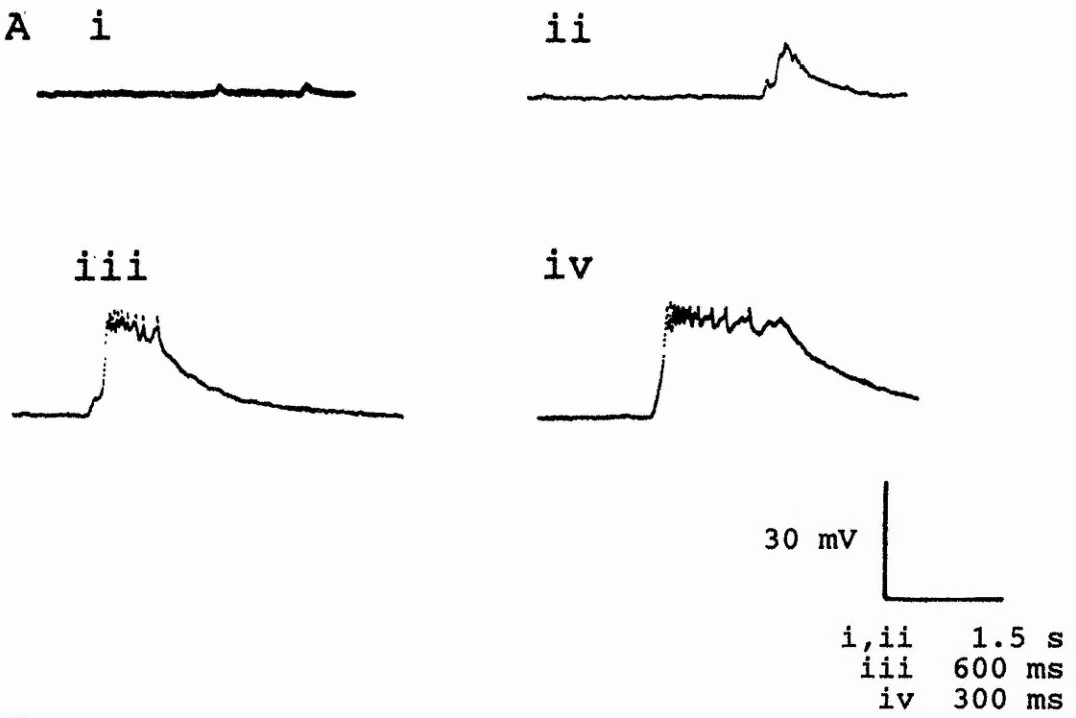
The irregularity of the PTX-evoked activity, together with the abrupt take-off of plateau potentials, suggests that the trigger eliciting spontaneous plateaux in the presence of this agent was excitatory synaptic drive. However, whilst changes in the resting conductance of D_f following PTX treatment were not monitored, PTX certainly exerted some direct action upon D_f in addition to any effects at the level of the synapse: individual plateau events were not followed by prolonged afterhyperpolarisations, as described for plateaux from the normal D_f (Fig. 3.5) (for examples: see Fig 3.32A_{iii}; B and C), suggesting that the repolarising currents were in some way directly affected by PTX.

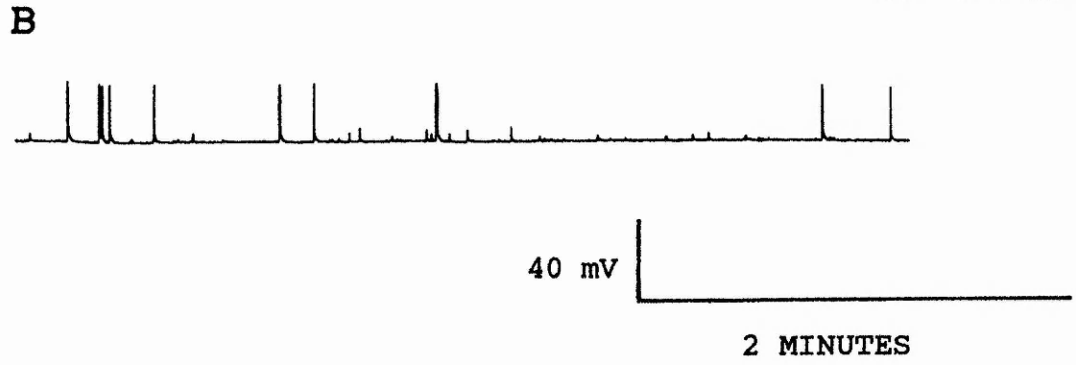
FIGURE 3.32: Effects of 10^{-5}M picrotoxin (PTX) on spontaneous electrical activity recorded from D_f.

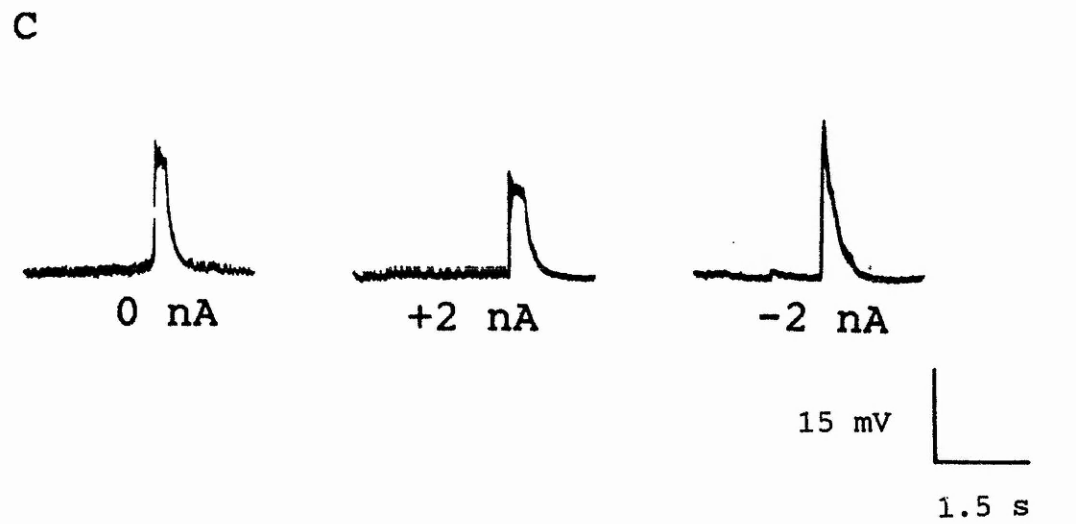
(A) (Ai) Spontaneous activity recorded from a neurone in normal saline. (Aii) Enhanced membrane potential fluctuation recorded from cell after 30 minutes exposure to PTX. (Aiii, iv) Spontaneous plateau potentials recorded from same cell after 41 and 45 minutes exposure to PTX respectively. Note that attenuated action potentials surmount the plateau phase of these events. Resting potential of this cell -71mV.

(B) Record of spontaneous picrotoxin-induced responses taken from a different preparation two hours after picrotoxin addition. Record was taken using a relatively slow time-base to illustrate the irregular nature of this spontaneous activity. Resting potential -73mV.

(C) Individual PTX-induced events from a third neurone at the resting potential (0nA) and under depolarising (+2nA) and hyperpolarising (-2nA) bias, illustrating that these events take-off abruptly from a 'steady' membrane potential. Resting potential of cell -70mV.


A
i
ii
iii
iv
30 mV
i,ii 1.5 s
iii 600 ms
iv 300 ms


B
40 mV
2 MINUTES


C
0 nA
+2 nA
-2 nA
15 mV
1.5 s

3.4.3. Pentylenetetrazole (PeTZ)

The observation of spontaneous plateau potentials in D_f following treatment with picrotoxin, which is a powerful convulsant, prompted further experiments using another convulsant agent. The convulsant chosen was pentylenetetrazole, a metrazole derivative (6,7,8,9-Tetrahydro-5H-tetrazolo-[1,5-a]azepine); one of a family of agents commonly used to induce epileptiform seizures in model systems used for the screening of antiepileptic drugs; and a drug of historic interest for those working on burst-generating mechanisms (for example: David *et al.*, 1974).

Pentylenetetrazole (PeTZ; this abbreviation has been chosen, rather than the usual PTZ, to avoid confusion with PTX) was added to preparations at a final bath concentration of 25mM (a similar concentration to that used in previous studies by David *et al.*, 1974; and Mc.Crohan and Gillette, 1988). In each of five preparations this agent induced regular, spontaneous plateau potentials within minutes of addition (Fig. 3.33). In contrast to PTX-induced activity, the development of spontaneous plateaux in PeTZ-saline was accompanied by a depolarisation of the resting potential; individual plateau potentials were also preceded by slow membrane potential depolarisations (Fig. 3.33B; Fig 3.34A). The onset of spontaneous plateau generation was rapid in comparison

with the action of PTX, in one case being as little as 30s. On washing the preparation in normal saline, neurones hyperpolarised towards their original membrane potentials and spontaneous plateaux were no longer evident (Fig. 3.33C) (although plateau potentials could be elicited using depolarising current injection, as in the untreated neurone); therefore, the effects of PeTZ appeared to be reversible.

The spontaneous plateau potentials recorded in PeTZ-containing saline closely resembled those in the untreated neurone: they were of similar amplitude, occurred beyond a certain threshold membrane potential (between -50mV and -60 mV), possessed distinct afterhyperpolarisations and attenuated spikes surmounting the plateau phase. The structure of the spike burst was similar following PeTZ exposure to that recorded in normal saline, although both instantaneous frequency and number of spikes within the burst were observed to increase; these effects were reversible on washout (Fig 3.34B).

The discharge pattern of spontaneous plateau potentials following PeTZ treatment was regular, in contrast with the irregular discharge recorded following PTX treatment (compare Fig. 3.34A with Fig. 3.32B). This suggests that pentylenetetrazole acted primarily upon D_f itself rather than upon synaptic inputs to it. Moreover, PeTZ almost certainly had direct actions on the membrane properties of D_f

because: (a) short (50 ms) depolarising pulses were sometimes able to elicit plateaux in the presence of PeTZ but not in standard saline (Fig. 3.35A) and (b) plateaux could be elicited using 'anode-break' following PeTZ treatment, but not in standard saline (Fig. 3.35B). Although measurements of effective membrane resistance and time-constant were taken in standard saline and in saline containing PeTZ, the membrane potential was constantly changing in the presence of the convulsant, and therefore it proved uninformative to make comparisons of these parameters since they could not be taken at predetermined values of membrane potential.

Whilst the above evidence suggests that PeTZ exerted effects directly on D_f, since it was bath applied it undoubtedly affected other neurones within the ganglion, possibly including neurones presynaptic to D_f. Accordingly, it is not possible to exclude synaptic actions of this agent without further experimentation.

FIGURE 3.33: Effect of 25mM pentylenetetrazole (PeTZ) on spontaneous activity in D_f. (A) Record of resting potential of cell in normal saline prior to PeTZ addition. (B) Spontaneous plateau potentials recorded after two minutes of PeTZ application. (C) Record of membrane potential after fifteen minutes washout of PeTZ. In each record a reference potential (-70mV) is marked with a dotted line.

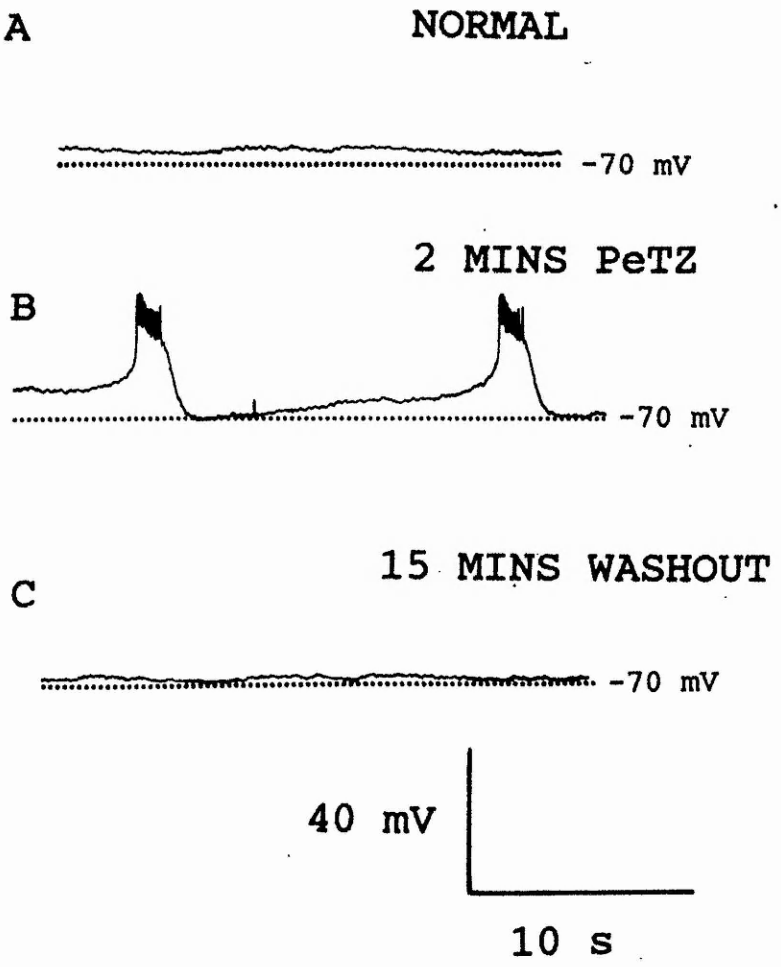

A
NORMAL
-70 mV
2 MINS PeTZ
B
-70 mV
15 MINS WASHOUT
C
-70 mV
40 mV
10 s

FIGURE 3.34: (A) Record of PeTZ induced plateau potentials (same cell as Fig. 3.33) recorded 9 minutes after PeTZ addition, showing the regular nature of the PeTZ induced activity.

(B) Analysis of effect of PeTZ on the structure of impulse bursts driven by plateau potentials (different cell from A). 'CONTROL' frequency-spike relationship (crosses) was constructed from an impulse burst driven by a plateau from a preparation in normal saline (plateau evoked by current injection). 'PeTZ' relationship (asterisks) was constructed for a spontaneous plateau recorded after several minutes exposure to 25mM PeTZ. 'WASHOUT' relationship (filled squares) was constructed for a plateau evoked by current injection after 10 minutes washout of PeTZ in normal saline.

A


-70 mV
40 mV
20 s

B

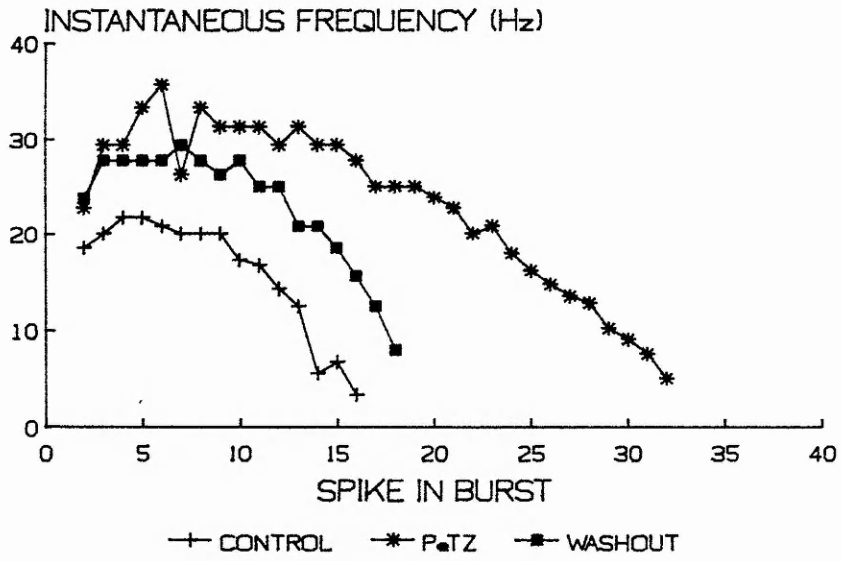
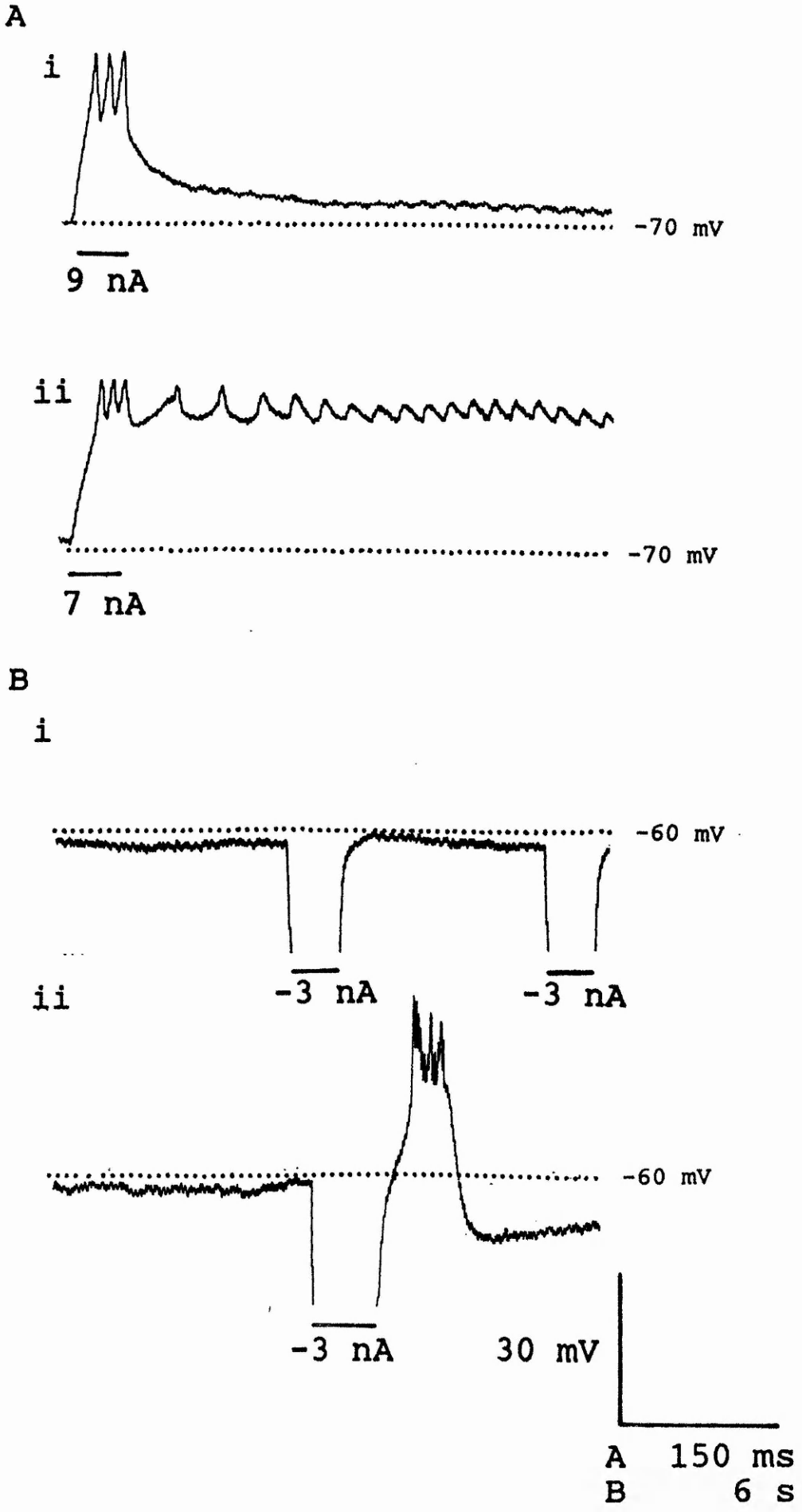

INSTANTANEOUS FREQUENCY (Hz)
40
30
20
10
0
0
5
10
15
20
25
30
35
40
SPIKE IN BURST
CONTROL
PeTZ
WASHOUT

FIGURE 3.35: Evidence that PeTZ affects intrinsic membrane properties of D_f.

(A) A brief depolarising pulse applied to a cell in normal saline evoked an oscillatory response. (Ai). A similar duration, but smaller magnitude pulse applied after the cell had been exposed to 25mM PeTZ produced an oscillatory response followed by a prolonged plateau (Aii).

(B) Relatively long duration hyperpolarising pulses applied to a different neurone failed to elicit a plateau potential by 'anode-break' (Bi), but could do so after the cell had been exposed to 25mM PeTZ (Bii).

For each cell a reference potential (A -70mV; B -60mV) is represented on each trace by a dotted line. Horizontal bars represent duration of applied current pulses.


A
i
-70 mV
9 nA
ii
-70 mV
7 nA
B
i
-60 mV
-3 nA
-3 nA
ii
-60 mV
-3 nA
30 mV
A 150 ms
B 6 s

3.5. Active properties of other insect neurones

Preliminary experiments were performed to determine whether any other insect neurones possess membrane properties which could mediate plateau-type events. Time constraints limited the search to one other cockroach neurone, and a motoneurone from the metathoracic ganglion of the locust.

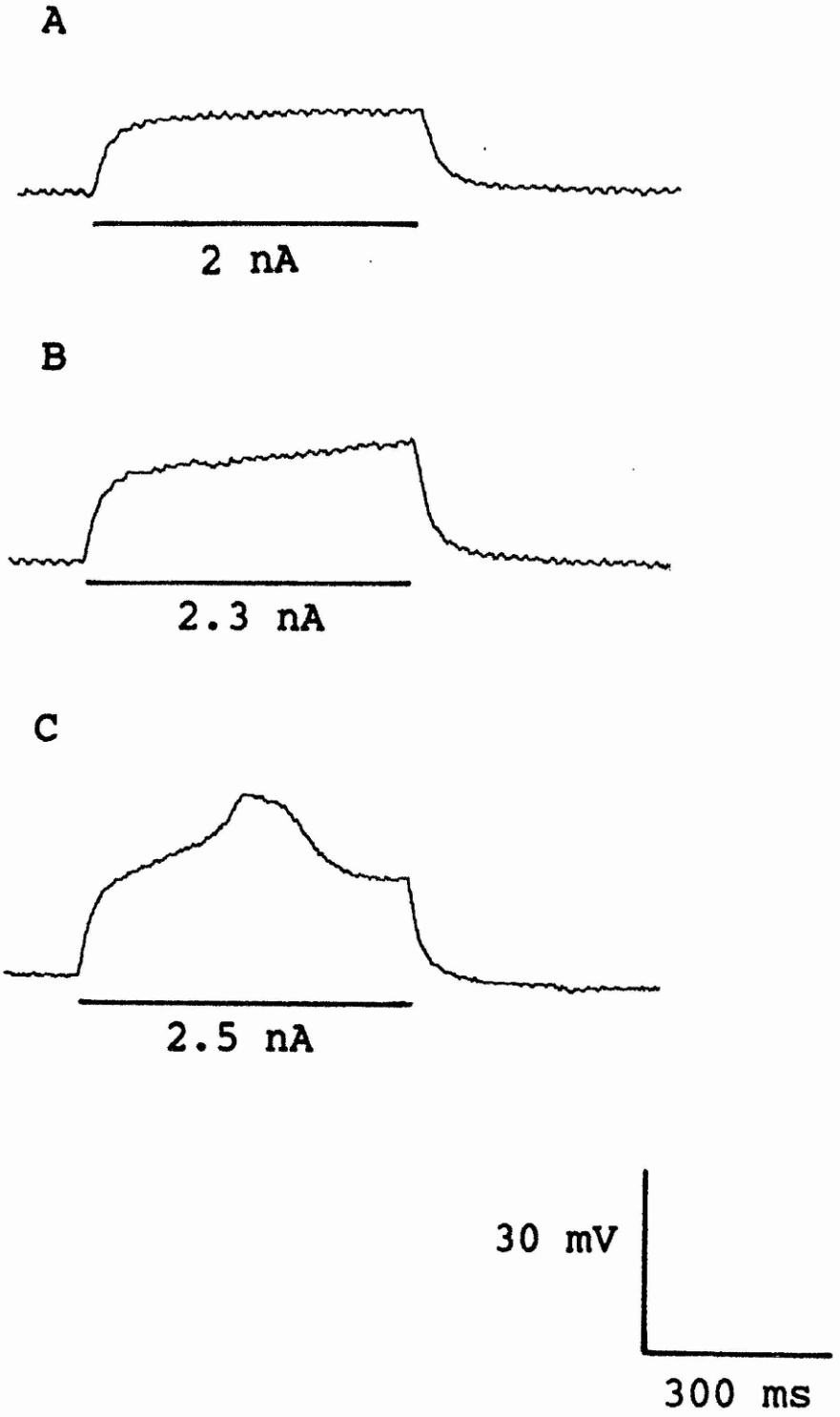
3.5.1. Cell 3

Cell 3 is a 'fast' motoneurone located in the cockroach metathoracic ganglion. It innervates the bifunctional basalar/coxal depressor muscle 177C (Carbonell, 1947). In flight this muscle causes wing depression (Fourtner and Randall, 1982) and during walking it causes extension of the femur (Fourtner and Randall, 1982). During walking cell 3 acts as a synergist of D_f. It has been shown to possess many membrane properties in common with D_f (Nightingale and Pitman, 1989) and, therefore, seemed an obvious candidate for investigation.

Fig. 3.36 shows the response of this neurone to 500ms depolarising current pulses of increasing amplitude. Beyond a threshold level, the cell responded in a non-linear fashion, exhibiting short duration plateau-type events. Although these events did not appear to outlive the applied pulse, their observation

demonstrated that, in common with D_f, this neurone possesses non-linear properties which have not been previously reported and, therefore, warrant further investigation.

FIGURE 3.36: Non-linear membrane properties of cell 3. Records of response of cell 3 to relatively long duration, small amplitude depolarising pulses of increasing intensity. Resting potential -68mV. Horizontal bars represent duration of applied current pulses.


A
2 nA
B
2.3 nA
C
2.5 nA
30 mV
300 ms

3.5.2. Locust 'fast' extensor tibiae motoneurone (FETi)

Similarly to D_f and cell 3, FETi is a 'fast' motoneurone. This neurone innervates the muscle providing the power for the locust jump (Hoyle and Burrows, 1973) thus its membrane properties have potential influence upon the generation of movement; in addition, its large size and accessibility made it an obvious candidate for study.

In a previous study upon the excitability of FETi a semi-intact preparation was used and this neurone was identified by recording antidromic axon-spikes evoked by stimulating the extensor tibiae muscle (Goodman and Heitler, 1979). The isolated ganglion preparation used for the present experiments precluded such a means of identification; FETi was identified from its distinctive responses to depolarising current pulses passed into the cell body and also to axonal stimulation via a suction electrode applied to N5. Antidromic stimulation of this neurone evoked an action potential in the axon which conducted electrotonically and decrementally back to the soma (Fig. 3.37A). The attenuated amplitude of this action potential is large (up to 30 mV) and , therefore, it is quite distinct from those recorded from other neurones in which the amplitude of such events is generally no greater than 5 mV. Application of a depolarising current pulse into the soma could also

elicit an action potential in the axon which propagated electrotonically back into the soma (Fig. 3.37B) and regeneratively down the axon (Fig. 3.37C).

Experiments were performed under conditions which might optimize the observation of plateau-type events: preparations were bathed in saline containing picrotoxin. Following PTX exposure each of four cells examined exhibited spontaneous electrical activity of the type illustrated in Fig. 3.38. Fig. 3.38A shows a record of spontaneous events recorded from FETi approximately 1 hr following treatment with PTX. Each event consisted of a relatively rapid depolarisation (approximately 10mV), upon which small spikes were superimposed (generally two or three), followed by a sustained depolarisation, after which the membrane potential began to repolarise. (This is shown more clearly in Fig 3.38B). These events were not followed by afterhyperpolarisations; each event possessed its own, depolarising afterpotential. These spontaneous, picrotoxin-induced events were relatively short in their duration (100 - 200 ms), making it very hard to measure relative conductance at different stages during each event. These events appeared to be absent in the untreated preparation and, similarly to D_f, the effects of PTX were irreversible on washout.

FIGURE 3.37: Identification of locust FETi. (A) Antidromic axon spike recorded in neurone soma as a result of application of 2V, 0.4ms stimulus applied to nerve 5. Stimulus artefact is marked with an arrow. (B) Antidromic axon spike recorded from the soma of same cell as (A) following intracellular depolarising current injection. (C) Simultaneous recordings from nerve 5 (trace i) and the soma (trace ii) of a different neurone demonstrate that the axon spike evoked by depolarising current injection propagates orthodromically and antidromically. The extracellularly recorded event shortly precedes that recorded from the neurone soma. Resting potentials (A,B) -60mV; (C) -55mV. Horizontal bars represent duration of applied current pulses.

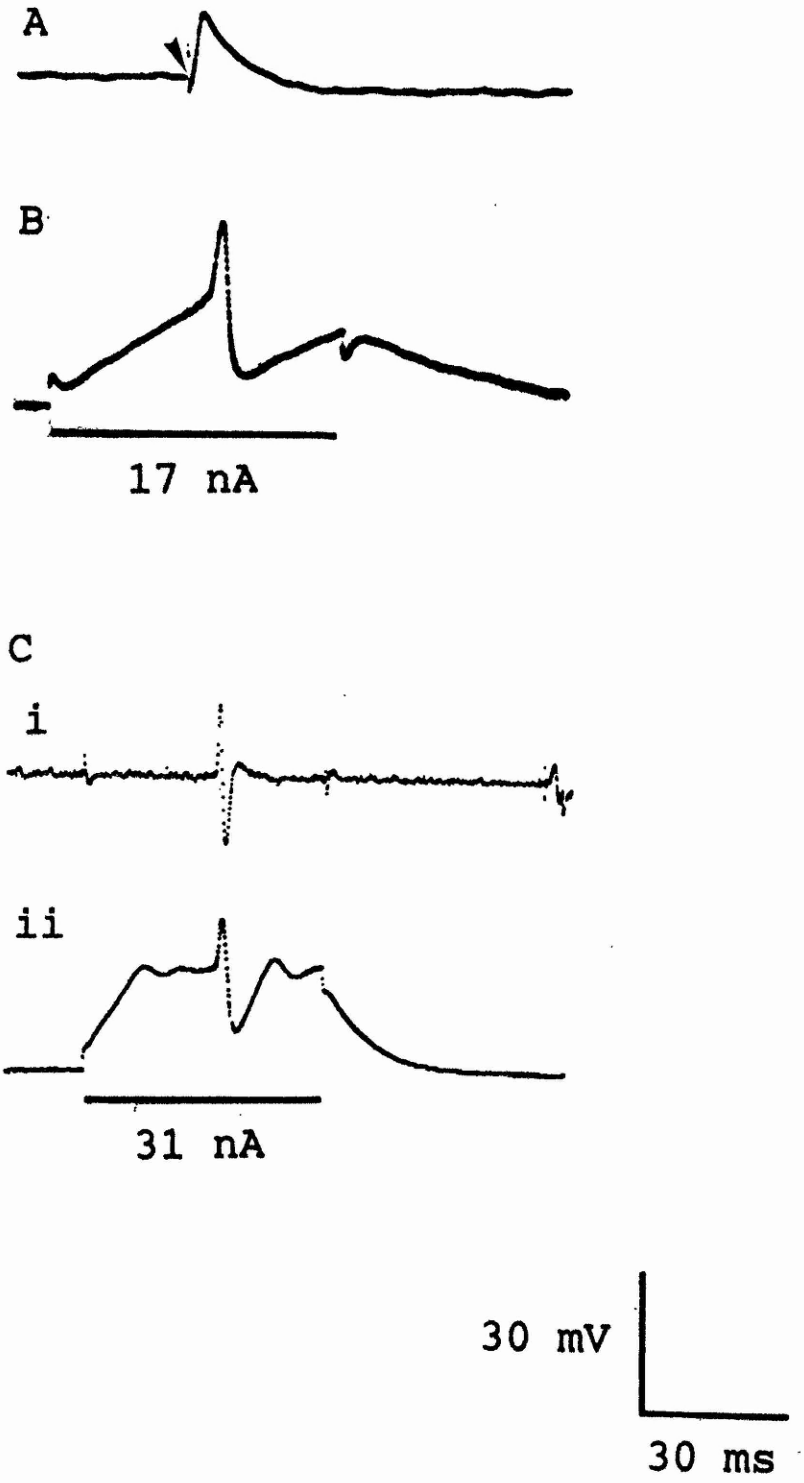
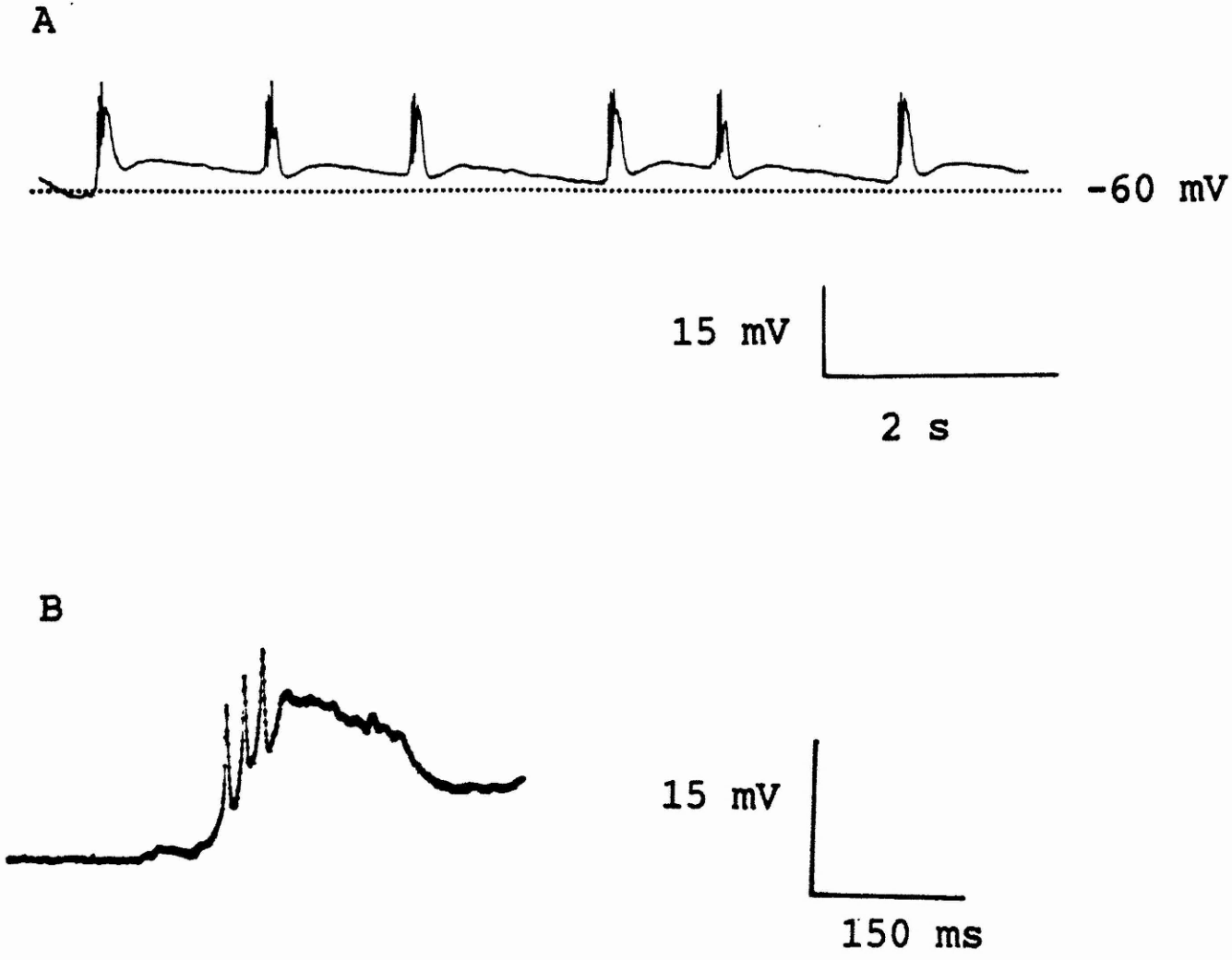

A
B
17 nA
C
i
ii
31 nA
30 mV
30 ms

FIGURE 3.38: Spontaneous depolarising events recorded from FETi approximately one hour after application of 10^{-5}M PTX. (A) Sample recording of a number of PTX-induced events; a reference potential (-60mV) is marked with a dotted line. (B) A single PTX-induced event (same cell as A) showing attenuated action potentials on the rising phase of the response.


A
-60 mV
15 mV
2 s
B
15 mV
150 ms

DISCUSSION

3.6. Characterizing plateau potentials in D_f.

The results presented here constitute the first evidence that insect neurones can possess burst forming capabilities in the absence of pharmacological treatment. Plateau potentials have been recorded from cockroach giant fibres following treatment with the potassium channel blockers TEA and 4AP (Yawo *et al*, 1985). However, these events differ from those observed in the present study in several fundamental respects: plateaux in giant fibres follow the sodium-dependent action potential and are of large amplitude (approximately 80mV); they are sodium-dependent; moreover plateaux have not been recorded from giant fibres without pharmacological intervention. Smaller magnitude, low-threshold, slowly-inactivating depolarising potentials were recorded from these neurones following partial blockade of potassium conductance by TEA alone. In the absence of TEA, however, the magnitude of the slow TTX-sensitive sodium current appeared insufficient to evoke plateaux.

Studies of the excitability of insect motoneurones have shown that action potentials with a long duration plateau phase can also be recorded from the locust FETi following blockade of some of the potassium conductance with barium ions, and that TEA

enhances the excitability of both cockroach and locust motoneurones (Goodman and Heitler, 1979; Pitman, 1979). However, slow regenerative events, such as those reported here, have not been recorded from such cells.

In contrast, the results presented here demonstrate that motoneurone D_f is capable of generating relatively slow depolarising potentials in response to appropriate stimulation. Whilst the cell responds to relatively brief depolarising pulses with a series of graded damped membrane oscillations (Figs. 3.2; 3.4A), it can respond to longer duration pulses with slower active events (Fig. 3.3). Given sufficiently long duration pulses, many cells examined were able to generate active depolarising events which could outlive the duration of the applied current pulse (3.4B). Since the current threshold for these events was consistently lower than that required to elicit damped membrane oscillations, these slow depolarising events are selectively activated by relatively long duration, low amplitude pulses.

These events satisfy the criteria for plateau potentials given by Russell and Hartline (1978) and Hartline *et al* (1988). That is to say:

(1) These events are active, possessing a distinct threshold (Fig. 3.4).

(2) In many instances, plateaux could outlast the duration of the depolarising pulses which evoked them (for example Figs. 3.4; 3.5) and, therefore, they are regenerative.

(3) In all experiments from intact neurones and neurones mechanically isolated *in situ*, plateau potentials could terminate spontaneously, but could also be prematurely terminated by application of a relatively brief hyperpolarising pulse (Figs. 3.9; 3.11).

(4) Experiments performed on mechanically isolated somata also demonstrated that these events can be recorded from neurones isolated from their synaptic inputs and, thereby, that these events result from activation of intrinsic membrane properties.

The active responses reported here, therefore, have been unequivocally demonstrated to be plateau potentials.

The plateau potentials observed in the present study appear similar to those recorded from neurones from crustacean stomatogastric and cardiac ganglia (Russell and Hartline, 1978; 1982; Raper, 1979; Tazaki and Cooke, 1979a). Most obviously, the duration and amplitude of cockroach plateaux are similar to those reported for plateaux from crustacean neurones and events recorded from the cell bodies of crustacean and cockroach neurones can be surmounted by attenuated action potentials. Similarities between crustacean

and insect events extend deeper than a mere visual resemblance, however, since the topographical organization of burst generating portions of the neurone appear to be similar for D_f and cardiac motoneurones (see below).

3.7. The topographical distribution of plateau- and impulse-generating membrane in D_f

3.7.1. Soma membrane contains plateau-generating machinery

Plateau potentials could be recorded from isolated somata (Fig. 3.11) which demonstrates that the soma of D_f is involved in plateau generation. However, since the plateaux recorded from isolated somata were (approximately) only a third as long-lasting as those from 'intact' neurones, it is likely that the plateau-generating portion of the neurone includes cell membrane from the initial segment which contains either an increased channel density or additional channel types. Furthermore, since plateaux in D_f can drive bursts of action potentials which appear to originate at some distance from the cell body (Fig. 3.7), it is probably necessary that plateau-generating membrane extends at least some way into the initial segment of the neurone.

That the soma of D_f contains plateau generating membrane points to a similarity between the events

recorded from this neurone and the 'driver' potential which drives bursting activity in cardiac motoneurones from the crab and lobster. Driver potentials in these neurones appear to originate from the soma and proximal dendrites of cells (Tazaki and Cooke, 1983a-c). Driver potentials were first revealed in crustacean neurones by treating cells with TTX; this had the effect of eliminating axonal impulse generation and highlighting events of somatic origin (Tazaki and Cooke, 1979c). The subsequent use of precisely positioned nerve-trunk ligatures confirmed that driver potentials could be recorded independently of axonal impulse generation and in the absence of incoming excitatory activity from other neurones, although the impulse-bursts were of shorter duration in the absence of chemically mediated excitatory input (Tazaki and Cooke, 1983b,c). Furthermore, ligaturing experiments demonstrated that the ability of cardiac motoneurones from *Homarus* to generate driver potentials is restricted to the soma and proximal axon and that approximately the first 1.2mm of proximal axon from these neurones does not support action potentials (Tazaki and Cooke, 1983b,c). For driver potentials to conduct to the axonal spike initiating zone (SIZ), it appeared that it was necessary for the driver potential to conduct along a portion of inactive cell membrane. Therefore, impulse-burst generation in these neurones involves electrotonic conduction of driver potentials to a distant impulse

generating zone (evidence reviewed by Benson and Cooke, 1984).

3.7.2. The impulse-bursts driven by plateaux originate in the axon

In the Tazaki and Cooke model of burst generation in cardiac motoneurones, driver potentials play a pivotal role in initiating bursts of action potentials in a distant part of the neurone (as discussed above). Driver potentials can conduct along non-spiking portions of the membrane and take the axonal spike initiating zone above the threshold required to initiate a burst of action potentials.

Simultaneous extra- and intra-cellular recordings from D_f indicate that plateau potentials in this cell can drive bursts of impulses which also appear to originate at some distance from the neurone soma (Fig. 3.7). Several lines of evidence support this. Firstly, plateaux from isolated somata are devoid of axon spikes, which suggests that the plateau-generating and impulse-generating portions of the neurone are spatially separate for this cell. Secondly, axonally generated action potentials reach extracellular recording sites before recording sites in the neurone soma (Fig. 3.7). Thirdly, the amplitude of axon spikes at the cell body is much attenuated (4-8 mV), compared to the soma spikes which can be induced by citrate injection or anoxia (Fig.

3.6). This last point suggests that the plateau- and impulse- generating portions of the neurone may be separated by an inexcitable portion of membrane, through which action potentials can only propagate passively. Whilst there is no direct evidence supporting this proposal, there is evidence that a portion of non-impulse-generating axon occurs in the locust 'fast' extensor tibiae motoneurone, FETi, (Gwilliam and Burrows, 1980). (This possibility means that location of the SIZ relative to the soma of D_f cannot be estimated from latencies between extra- and intracellularly recorded events since the axon spike may not propagate with similar conduction velocities in the anti- and orthodromic directions.)

Therefore, in D_f it is possible that orthodromic plateau conduction to the axonal SIZ involves electrotonic conduction through electrically inactive portions of the cell, just as burst generation in crustacean cardiac neurones involves spatially separate plateau- and impulse-generating sites; although, as yet, it is not possible to estimate how far from the soma the ability to generate plateaux extends. Clearly, if impulse generation in D_f is driven by plateau potentials which conduct along electrically inactive membrane, the decremented amplitude of plateaux at the axonal SIZ must be sufficiently large to drive axonal action potential bursts.

3.7.3. Topographical organization of burst-generating machinery: a *caveat*.

There appear to be differences in topographical organization of plateau generating membrane between crab and lobster cardiac neurones and some lobster stomatogastric (STG) neurones, in which the plateau generating portions of the cell may be located some distance from the soma (Russell and Hartline, 1982; Hartline et al, 1988). Some inter-neuronal differences in cellular properties, therefore, exist between different populations of burst-generating lobster neurones. By inference, it does not necessarily follow that, should plateau potentials be discovered for other insect neurones (preliminary evidence suggests that this is highly likely: Figs 3.36; 3.38), the burst generating machinery will be topographically located as described here for D_f.

3.8. Driver potentials in D_f?

Two factors force a comparison between the plateau potentials recorded from the surgically isolated cell body of D_f and those recorded from crustacean cardiac neurones after ligation of their axons. Firstly, the two events appear to originate in the proximal neurone. Secondly, both events drive impulse generation in electrically distant parts of the cell. Are the events directly analagous, or are they

phenomenologically different events which perform a similar function in these two neurone-types?

In phenomenological terms, the plateau potentials recorded from D_f-somata, mechanically isolated *in situ*, more closely resemble the appearance of the plateaux which are recorded from the 'intact' D_f than crustacean driver potentials, recorded following ligation, resemble the events in untreated cardiac motoneurones. The events recorded from insect somata, although considerably foreshortened, do possess a distinct plateau phase. On the other hand, crustacean driver potentials possess no plateau phase and, as a consequence, can only drive relatively brief bursts of impulses in the absence of excitatory synaptic drive from small-cell neurones (Tazaki and Cooke, 1983c). Furthermore, for D_f, the durations of plateaux recorded from intact neurones in response to a depolarising pulse appear similar in normal saline and saline containing TTX at concentrations sufficient to block axonal impulse production (Fig. 3.21). This contrasts with the effects of TTX on cardiac motoneurones, where plateaux undergo a gradual decline in duration with exposure, before giving way to the underlying driver potential (Tazaki and Cooke, 1983c). As a consequence, whilst driver potentials are responsible for initiating bursts of axonal impulses in cardiac neurones, burst duration depends upon an interaction between driver potentials and incoming excitatory synaptic influences (Tazaki and Cooke,

1983a-c; Benson and Cooke, 1984). That the events recorded from the intact D_f exposed to TTX and from isolated D_f somata possess a distinct plateau phase, may indicate that excitatory synaptic influences are not as important in maintaining burst duration in D_f as in cardiac motoneurones. However, although the synaptic inputs to motoneurone D_f are unknown, the interruption and premature termination of plateaux by hyperpolarising current pulses (Figs. 3.9, 3.11) does suggest that the structure of these events is susceptible to alteration.

In functional terms, the available evidence points to similar roles for somatic plateaux from D_f and crustacean driver potentials. It is certainly possible to propose a similar spatial integration of events in D_f leading to the plateaux recorded from the 'intact' neurone, to that put forward for cardiac motoneurones (see section 1.4.2). However, it must be stressed that whilst plateau potentials can be recorded from the isolated soma of D_f this does not mean that the cell body, *per se*, is the primary site of plateau production in this cell under physiological conditions.

It is possible that it is the initial segment which is the primarily site of plateau generation in D_f and that the ability of the cell body to produce plateaux merely reflects the presence of redundant channels in the neurone soma. However, an alternative

possibility is that plateau generation by the soma is important in ensuring orthodromic plateau conduction and not merely the result of some overspill of channels into the cell body. The large size of the D_f soma (often in excess of 80µm) and its electrical proximity to the initial segment of the neurone mean that, in the absence of any plateau-generating mechanism, it has a huge potential as a current-sink. A passive soma would extinguish a plateau before it could conduct orthodromically to the impulse-generating portion of the axon. In contrast, by possessing channels capable of generating plateaux, the cell body would respond regeneratively to an event arising in the initial segment. This would allow the soma to assist the initial segment in plateau generation, rather than drawing current into passive membrane, thereby allowing the event to conduct orthodromically and ensuring that the amplitude of the plateau at the axonal SIZ is sufficiently large to elicit an impulse-burst.

3.9. Action potential burst organization differs between D_f and some molluscan bursters.

The independence of plateau potential production from axonal impulse generation in D_f contrasts markedly with the interaction between action potentials and burst-generating potentials that occurs in some molluscan bursting neurones. In these cells currents activated by action potentials appear to play active roles in sustaining and terminating bursting activity (Lewis, 1984; Kramer and Zucker, 1985a,b; Adams, 1985; Adams and Levitan, 1985; Adams and Benson 1985; 1989). In contrast, plateau potentials devoid of superimposed axon spikes could be recorded from the 'intact', untreated D_f. These plateaux were of similar amplitude and duration to those driving axonal impulse-bursts, suggesting that impulse-activated currents are not required to sustain plateaux in this neurone. Furthermore, the duration of plateau potentials in D_f was little affected by application of TTX, even though this blocked axonal impulse generation (Fig 3.21).

In molluscan pacemaker neurones such as R_{15} from *Aplysia* the distinct 'parabolic' shape of the burst results from the interaction of depolarising and hyperpolarising transient currents activated by the action potentials which form the burst (see section 1.4.1.B-D). The situation for D_f may differ from that for neurones such as R_{15}, since, in the apparent absence of any impulse-driven currents, the impulse-

burst driven by plateaux in D_f does appear to possess a qualitatively similar structure from neurone to neurone (Fig. 3.8). Presumably this results from the structure of the plateau itself when it arrives at the axonal SIZ. Thus, it might not always be necessary to invoke a strong interaction between a burst-forming potential and action potentials within a burst in order to produce a relatively structured bursting output.

3.10. Membrane properties underlying plateaux in D_f.

Data from current-clamp experiments indicates that plateau potentials in D_f result from the activation of a relatively slow inward current. Plateaux from intact neurones and isolated cell-bodies are associated with a high membrane conductance at the crest of the plateau phase relative to that at sub-threshold membrane potentials (Figs. 3.12, 3.13). This shows that plateaux are associated with activation of an inward current. Clearly, since plateaux are selectively activated by relatively long duration pulses, this inward current is relatively slow in its activation. Moreover, since the increase in membrane conductance during plateaux declines during the course of these events (Fig. 3.14), some inactivation of the inward current appears to occur. This contrasts with observations made for some molluscan neurones where the slow inward current driving bursting may be non-inactivating (Eckert and Lux, 1975; 1976), but corresponds to observations made for some crustacean and vertebrate neurones in which the inward current carrying plateaux can inactivate (Tazaki and Cooke, 1990; Legendre *et al*, 1988).

Data from voltage-clamp experiments supports the above observations made under current-clamp. Initial experiments, using a short duration pulse protocol, failed to uncover membrane properties which could be

responsible for plateau generation in D_f (Fig. 3.15); whilst subsequent experiments using a long duration pulse protocol revealed a negative resistance characteristic of neurones which could produce plateaux (Figs. 3.16-3.18).

For cells exhibiting plateau potentials, the I-V relationship contained a region of negative slope resistance (NSR) (Fig. 3.17). This region of NSR occurred in the membrane potential range between -60mV and -40mV, which correlates with the potential range over which plateaux occur. It was most evident for I-V curves constructed from currents sampled at later times into command steps (600ms); associated current records demonstrated that the region of NSR in the I-V relationship is associated with voltage-dependent activation of a slowly developing inward current. This inward current was absent from neurones which did not exhibit plateaux (Fig. 3.16). The magnitude of the slowly developing inward current did vary from preparation to preparation; whilst in some neurones it was of relatively small magnitude, in other instances neurones could develop a net inward current (compare Fig. 3.16B and Fig. 3.20).

The slow inward current was sensitive to stimulation frequency. I-V relationships from neurones differed depending on the delay between successive command steps; the I-V relationship became irregular if the delay between successive pulses was too short (Fig. 3.19). This appeared to result from

the failure of some command steps to activate an inward current at relatively higher stimulus frequencies, indicating that the inward current carrying plateaux requires some time to recover from inactivation. Indeed, the inward current generated by a regularly applied, suprathreshold command step could be eliminated if the delay between successive pulses was shortened (Fig. 3.20). Thus, not only does the slow inward current appear to inactivate, but it appears to be relatively slow to de-inactivate. A similar observation has been made for crustacean cardiac motoneurones: driver potential amplitude depends on stimulation rate, whilst the underlying inward calcium current is relatively slow to de-inactivate (Tazaki and Cooke, 1990).

The presence of a region of NSR in the I-V relationship of D_f is fundamental to its capacity to produce plateaux. The I-V relationship of D_f differs from that recorded from endogenously bursting molluscan neurones, however, since molluscan neurones show a continuous flow of inward current at negative potentials, and, therefore, the current in the I-V relationship for these cells lies below zero until relatively positive voltage (Wilson and Wachtel, 1974; Smith, Barker and Gainer, 1975) (see also section 1.3). In contrast, in the range of membrane potentials investigated here (between -70mV and -20mV), the I-V relationship of D_f lies positive to

the zero current (voltage) axis; the region of NSR only transects this axis for cells capable of producing a net inward current (for example Fig. 3.19Ai, Bi). In this respect the I-V curve of D_f resembles that recorded for lumbar motoneurones from the cat (Schwindt and Crill, 1977). This feature of the I-V relationship means that although D_f and cat-motoneurones do not usually produce spontaneous impulse-bursts (as do some endogenous molluscan bursters such as R_{15}), any stimulus which shifts the membrane potential into the NSR-voltage-range can drive a relatively long-lasting impulse-burst. This has functional implications in terms of the role bistable properties may play in locomotor motoneurones: such properties may be required to alter the gain of excitatory synaptic input, acting to enhance the intensity and/or duration of the post-synaptic response; although the locomotor rhythm itself is centrally generated (Delcomyn, 1980). In contrast, spontaneously bursting neurones can play key roles in central rhythm generating networks and therefore require membrane properties allowing them to produce impulse-bursts in the absence of patterned excitatory synaptic drive.

3.11. Ionic basis of plateau potentials.

3.11.1. Plateau potentials are predominantly calcium-dependent

A large portion of the inward current generating plateaux in D_f is carried by calcium ions. Somatic plateaux from D_f appear to possess a similar ionic dependence to crustacean cardiac motoneurone driver potentials, which are sodium-independent and sensitive to Cd^{2+} and Mn^{2+} ions (Fig. 3.24; Tazaki and Cooke, 1979c; 1986; 1990).

Plateaux from the intact D_f also appeared sensitive to Cd^{2+} ions (Fig. 3.22), although blockade was considerably slower in onset and less readily reversible than that observed for isolated somata. In addition, the region of NSR in the I-V relationship of intact cells could be eliminated by Mn^{2+} ions (Fig. 3.23). These observations indicate that a sizeable portion of the current carrying plateaux in intact neurones is carried by calcium ions and that the slow current responsible for the NSR in the I-V relationship is Ca-dependent. A similar observation has been made for the NRC of bursting neurones from *Helix* (Eckert and Lux, 1976). However, at present, precise information concerning activation and inactivation kinetics of the slow inward current in D_f is unavailable; neither is it possible, as yet, to

state whether inactivation of this current is Ca- or voltage-dependent.

However, two experimental observations suggest that the ionic dependence of plateaux from the intact D_f may be more complex than that for the isolated cell body, involving an additional ion (or ions) as a carrier of inward current:

(1) Plateaux from intact neurones are of longer duration than those from isolated cell bodies (approximately three times as long in duration).

(2) The slow onset of the effects of Cd^{2+} and Mn^{2+} ions on intact cells relative to isolated somata.

Observation (1) can be accounted for if calcium channels in the initial segment of the neurone differ from those in the soma (either in their density or inactivation kinetics), or, alternatively, by the involvement of an additional channel type to calcium in plateau production by intact cells. Observation (2) could be explained if access to channels carrying Ca-current underlying plateaux was easier following the isolation procedure than in the intact preparation; but could also result from a 'mixed' ionic dependence of plateau potentials in intact cells.

Thus, it is possible that a portion of the inward current carrying plateaux in the intact neurone is carried by a second ion, most probably sodium. Any

sodium-dependent component must be TTX-insensitive, however (Fig. 3.21), and could only be unequivocally demonstrated using ion-sensitive dyes. (TTX-insensitive, sodium-dependent plateaux have been recorded from vertebrate motoneurones (Wallén and Grillner, 1987). Sodium-substitution experiments could give equivocal results because of possible access problems in the intact preparation, requiring long periods of exposure to ensure that changes in the perfusate reach the microenvironment of the cell. (Ion substitution experiments have proved difficult in previous studies of this neurone by Thomas (1984), Pinnock *et al* (1988) and Nightingale and Pitman (1989). Prolonged exposure to low-sodium saline is undesirable because of secondary effects of (membrane hyperpolarisation and a fall in effective membrane resistance) which could be due to effects on a Na\Ca exchange mechanism. (Interestingly, little evidence exists for such an exchange mechanism in insect neurones and, therefore, this could prove a fruitful avenue for research.)

In the anterior burster (AB) neurone of the lobster different bursting mechanisms mediated by sodium or calcium ions can be differentially activated by different neurotransmitters (Harris-Warrick and Flamm, 1987) and plateau potentials in cerebellar Purkinje cells can be mediated by sodium and calcium ions in the same cell (Llinás and Sugimori, 1980). Moreover, whilst Tazaki and Cooke have established

that the current underlying driver potential production in 'large cell' cardiac motoneurones is carried by calcium ions (Tazaki and Cooke, 1986; 1990), there is some evidence to suggest that sodium ions are involved in driver potential production in the smaller 'pacemaker' cells (Berlind, 1985). Therefore, it is conceivable that both sodium and calcium ions can play a role in generating plateaux in D_f. The present results, however, do suggest that the majority of the inward current is calcium-dependent.

3.11.2. The evidence for two types of calcium channel in D_f

To date, little information concerning the nature of calcium channels in insect neurones has been obtained. Calcium channels have been demonstrated in cultured cockroach neurones, using whole cell patch-clamp (Christensen *et al*, 1988) but no differentiation of these channels into sub-types has been made. Calcium channels can mediate action potentials in D_f following pharmacological manipulation (Pitman, 1979). The present data suggest that plateau potentials in D_f are mediated by a different class of Ca-channel to those which carry the current of citrate-induced action potentials (Figs. 3.25-3.30). The evidence for this is as follows:

(1) Citrate-dependent action potentials can be activated by short-duration current pulses (Fig.3.25).

Plateaux cannot be similarly activated, suggesting differences in the activation kinetics of the calcium channels involved in the two events.

(2) Plateau potentials and citrate-dependent action potentials can be recorded from the same cell simultaneously (Fig. 3.26). Citrate-spikes and plateaux also have very different time-courses and attain different peak amplitudes. These observations indicate that plateaux do not result from changes to channels involved in carrying action potentials, or *vice versa*.

(3) I-V relationships of cells demonstrating plateaux and citrate-spikes contain two distinct regions of NSR which can be associated with citrate-spikes and plateaux (Figs 3.27-3.30). Currents sampled early into command pulses produce an I-V curve with a NSR associated with citrate spikes. Currents sampled later into pulses produce a NSR which is associated with plateaux. These two regions of NSR have different voltage-dependencies.

Thus, plateaux in D_f do not result from pathological or activity-induced changes to 'fast' calcium channels. In some molluscan cells repetitive stimulation can convert calcium-spikes into depolarised plateaux lasting for a number of seconds, probably as a result of frequency induced inactivation of K^+ currents and Ca^+ induced inactivation of I_C (Pin *et al*, 1990; Crest *et al*, 1990). It is not possible

to invoke a similar mechanism here since spikes and plateaux can co-exist. Therefore, the available experimental evidence indicates that there may be more than one class of calcium channel present in the cell membrane of motoneurone D_f.

These observations parallel those taken from experiments upon turtle lumbar motoneurones (Hounsgaard and Kiehn, 1989). Plateau potentials from turtle motoneurones are sensitive to nifedipine and enhanced by the L-channel agonist Bay K 8644, but fast TEA-induced action potentials are insensitive to this agent. Therefore, plateau potentials in turtle motoneurones appear to be mediated by channels belonging the slowly inactivating L-class, whilst a different class of Ca-channel can carry action potentials in the presence of TEA.

It is not known whether invertebrate calcium channels can be categorized according to the sub-classes drawn up for vertebrate Ca-channels. Indeed vertebrate Ca-channels themselves can possess properties of more than one class of channel (Triggle, 1989); and whilst L-channels appear to mediate plateau potentials in turtle motoneurones (Hounsgaard and Kiehn, 1989), T-channels may also be important in the development of bursting activity in some vertebrate neurones (Suzuki and Rogawski, 1989; White *et al*, 1989; Lovinger and White, 1989). Calcium channels mediating plateaux in D_f are activated at negative

voltages (similarly to T-channels), but may possess relatively slow inactivation kinetics and appear to be Cd^{2+}-sensitive (similarly to L-channels). Further experiments are required to characterize these channels.

The present data do not indicate whether the slow afterhyperpolarisation which follows plateaux in D_f results from inactivation of channels carrying inward current during these events, such as that proposed for R_{15} from *Aplysia* (Adams and Benson, 1989), from calcium-dependent activation of potassium conductance, or a combination of both of these. In view of the many similarities between crustacean driver potentials and plateaux in D_f it is not unlikely that the post-plateau hyperpolarisation results from activation of I_C; although a combination of I_C-activation and slow de-inactivation of slow inward current probably combine to determine the refractory period following an impulse-burst.

3.12. A model for plateau potential generation in D_f.

Putting together the various pieces of information from current- and voltage-clamp experiments, it is possible to propose the series of ionic events which lead to plateau production in D_f. In the following scheme, certain steps are proposed from a sound experimental basis; others are included for the sake of completeness and require experimental verification.

Starting at the beginning of a plateau potential, the event ensues once a depolarising influence (resulting from either current injection or excitatory synaptic input) activates the slow inward current which carries the rising phase of the plateau. Due to its regenerative nature (shown by voltage-clamp analysis) activation of this current accelerates and calcium ions move into the cell, depolarising the membrane potential. However calcium entry also activates the calcium-dependent potassium current (Thomas, 1984), whilst the outward current responsible for the delayed potassium current of D_f may be activated by excursions of the membrane potential positive to -40mV. Thus a balance exists between net inward and outward driving forces. Initially the inward driving force (provided by the slow inward current) predominates; but as the intracellular calcium concentration rises the activation of I_c increases, and the slow inward current itself begins to inactivate. Once the sum of the net outward

currents exceeds the magnitude of the slow inward current, the plateau phase of the response is terminated and the cell hyperpolarises. As a result of the relatively high level of intracellular calcium (carried by the slow inward current), I_c is strongly activated and the membrane potential strongly repolarises towards and beyond the original resting membrane potential of the cell, resulting in the afterhyperpolarisation which follows plateaux. At this stage, the cell cannot respond to an incoming excitatory stimulus because the slow inward current is slow to de-inactivate; this determines the absolute refractory period following plateaux. In addition, of course, the relatively high outward conductance (resulting from I_c) and relative negativity during the after hyperpolarisation determine the relative refractory period. Once the slow inward current has recovered from inactivation, it can be reactivated. As the concentration of Ca^{2+} falls inside the cell, I_c decreases and the cell repolarises. During this stage, an incoming excitatory stimulus (of sufficient magnitude/duration) can activate the slow inward current and, thereby, evoke a plateau.

3.13. Manipulating plateau potentials in D_f.

Bath applied GABA was effective in eliminating plateau potentials in D_f; this was associated with an increase in the membrane conductance of the neurone (Fig. 3.31). GABA receptors have been identified and characterized for the cell body of D_f; activation of these receptors appears to result in an increase in permeability which is mediated primarily by chloride ions (Sattelle *et al*, 1988; Pinnock *et al*, 1988). The sensitivity of plateau potentials to increases in membrane conductance produced by GABA offers a way in which the neurone can be inhibited from expressing these events. Conductance changes can have profound effects on the ability of neurones to generate bursts: in a neurone from *Helix*, a decrease in resting conductance induced by firing of a presynaptic neurone induces or enhances bursting activity (Pin and Gola, 1987). In the lobster pyloric system, both exogenously applied GABA (10^{-4}M) and stimulation of GABA-ergic input fibres can suppress oscillatory activity (Cazalets *et al*, 1987).

Insect GABA receptors appear to share some common characteristics with vertebrate $GABA_A$ receptors, and the GABA response in D_f is sensitive to picrotoxin which is believed to act non-competitively, blocking the GABA-gated chloride channel, and/or potentiating the closed state of the receptor (Pinnock *et al*, 1988; Lummis, 1990). Picrotoxin application was able to

induce spontaneous plateaux in D_f. The irregularity of these spontaneous events and lack of consistent changes in resting membrane potential suggest that spontaneous plateaux result from changes to the synaptic influences acting upon the cell (Fig. 3.32). However, since membrane conductance was not monitored in this series of experiments it is not possible to state that picrotoxin had no direct effects on the membrane of D_f. Indeed, plateaux in the presence of this agent often lacked the prolonged afterhyperpolarisations which are characteristic of these events, suggesting a direct action of picrotoxin on channels involved in the repolarisation phase of the event. Consistent with this, it has been reported previously that the actions of picrotoxin are not confined to receptor-activated chloride channels in D_f, but may extend to channels carrying potassium ions (David and Sattelle, personal observations; cited from Sattelle *et al*, 1988). However, if PTX acts primarily by blocking postsynaptic GABA receptors it is possible that tonic GABA-ergic inhibition of excitatory inputs to D_f normally acts to prevent the cell from producing plateaux by presynaptic inhibition.

However, for D_f it is unlikely that the expression of plateau potentials depends simply on modulation between states of high and low membrane conductance, since cells which did not exhibit plateaux often possessed similar input resistances to those which

could express these events. Likewise, the I-V relationships of plateau-generating and "passive" (non-plateau-generating) cells generally differed only over the range of potentials over which the region of NSR is expressed (Fig. 3.17). This suggests that the ability to generate plateaux is modulated in a more subtle fashion than gross changes in conductance: agents acting in a voltage-dependent manner may be able to switch the cell between plateauxing and non-plateauxing modes. Although preliminary investigation was made to determine whether a range of neuromodulatory substances could modulate the ability of D_f to generate plateaux, proctolin, dopamine, glutamate and the muscarinic agonist NcN A343 yielded variable results. Therefore, future attention must focus on these and other potential modulators of plateau potentials in this cell.

D_f does appear to possess membrane conductances which are susceptible to modulation, however, as evidenced by the actions of the convulsant pentylenetetrazole (Figs. 3.33-3.35). In the presence of this agent, regular, spontaneous plateau potentials could be recorded from D_f. Individual events were followed by periods of hyperpolarisation, these in turn were followed by a pacemaker-like depolarisation towards the threshold for a successive plateau. The regular nature of this spontaneous activity suggests that PeTZ exerted its effects by direct actions on D_f.

Moreover, plateaux could be elicited in cells by relatively brief excitation in the presence of PeTZ and plateaux could be elicited on 'anode break' in the presence of PeTZ, but not in the untreated neurone (these observations demonstrate that PeTZ exerted direct effects on membrane properties of D_f).

PeTZ induces bursting in normally non-bursting cells from *Aplysia* by the induction of a region of NSR in the I-V curve (David *et al*, 1974). In snail neurones PeTZ is believed to release calcium from intracellular granules; this concentrates on the internal surface of the cell membrane, modifies ion channels and induces bursting activity (Sugaya and Onozuka, 1978a,b; Sugaya *et al*, 1987). Patch-clamp studies on cortical neurones have shown that changes in the open-close state of single ion channels occur following PeTZ treatment (Sugaya *et al*, 1989). The effects of PeTZ on D_f are probably more complex than an increase in intracellular free-calcium alone, however, since this would tend to decrease the excitability of D_f by enhancing I_C.

In the presence of PeTZ, the membrane potential of D_f is constantly cycling (there is no stable 'resting' potential in the presence of PeTZ). This suggests that PeTZ may shift the I-V relationship below the zero-current axis over a range of membrane potentials including the resting potential (for the untreated cell) and the potential range over which plateau potentials occur. It could achieve this, for

example, by enhancing an inward leak current, thereby providing the constant inward driving force required to drive the membrane potential into the activation region for the slow inward current. This effect may (or may not) combine with a suppression of outward conductances which are present in the untreated cell to produce spontaneous bursting in D_f. Further experiments may give deeper insights into the mode of action of PeTZ on D_f.

3.14. Possible reasons why previous studies have not revealed plateau potentials in insect neurones.

The fact that this study has uncovered plateau potentials in motoneurone D_f raises the question of why these events have not been reported before for this neurone, or indeed for other insect neurones. There are a number of possible reasons for this.

Current-clamp studies centring on this neurone have focused upon events which may be elicited by relatively brief stimulus regimens (Pitman *et al*, 1972b; Pitman 1975; Pitman, 1979; Pitman, 1988), whilst comparable voltage-clamp studies have utilised relatively short-duration clamp commands to minimise the build-up of ions in restricted extracellular spaces (Thomas, 1984; Nightingale and Pitman, 1989). Other studies have focused on transmitter activated currents (for example: David and Sattelle, 1984; Satelle *et al*, 1988). The results presented here

indicate that relatively slowly activating non-linear membrane properties are selectively activated by long pulse protocols; under current- and voltage-clamp short pulse protocols do not activate regenerative depolarising events (Figs. 3.2; 3.4A; 3.15). Therefore, since plateau potentials do not normally occur spontaneously in D_f, the experimental protocol is crucial in determining whether or not these events can be recorded from this cell.

Another plausible reason that these events have not been reported for D_f is that the recording conditions may have improved since early studies, due to improved oxygenation of the isolated ganglia preparation (see methods, section 2.2.1). Consistent with this proposal, values for effective membrane resistance and membrane time constant reported here are considerably higher than those reported by Pitman (1979) where a mean resting membrane resistance of cells of less than 2MΩ was reported. A recent study has shown that both the effective membrane resistance and resting potential of D_f are highly sensitive to oxygenation (David and Sattelle, 1990). In the present study, plateau potentials in D_f could be reversibly abolished by decreasing effective membrane resistance with GABA (Fig. 3.31), which suggests that these events are sensitive to changes in membrane conductance. Therefore, it is unlikely that early experiments on the excitability of this neurone would have observed plateaux, even in cells exposed to

sustained depolarising current injection, since the resting conductance of the neurones examined was generally high, thereby rendering cells incapable of generating these events. Isolated nerve cords from other animals have also been observed to exhibit increased activity as recording conditions have been improved. For example, this has proved important in establishing the concept of central control of rhythmic movement generation: whilst early studies failed to reveal rhythmic activity in isolated nerve cords from annelids, subsequent studies were successful in so doing (see Delcomyn, 1980 for review).

In more general terms, it is conceivable that non-linear, regenerative events have been present in insect neurones previously studied, but have been overlooked since other cellular or circuit properties have formed the focus for experimentation. Considerable precedent for this exists: plateau properties had been overlooked in lobster stomatogastric (STG) neurones until cellular properties received specific examination (Russell and Hartline, 1978; Raper, 1979).

3.15. The potential significance of plateau potentials in D_f to motor function.

The fact that D_f can produce plateau potentials implies that such events could participate in structuring motor output under certain circumstances. However, the precise role that D_f plays during normal walking is not known. Studies of insect locomotion have indicated that at slow walking speeds only 'slow' axons are recruited. As the frequency of stepping increases the frequency of impulse firing in any given axon increases whilst the burst duration decreases; and 'fast' axons are recruited. At very high speeds 'fast' axon activity may entirely obscure accompanying 'slow' activity (for a review see Delcomyn, 1985). Thus, it seems that it is the slow coxal depressor motoneurone, D_s, which is most active in driving hind-leg extension during normal walking. D_f may play a more prominent role at higher leg-speeds; it has been suggested that this 'fast' motoneurone may be involved in the escape response of the animal (Pearson and Iles, 1970). The consensus view is that the rhythmic firing of locomotor motoneurones is consequent upon a centrally generated oscillatory drive (Delcomyn, 1980) generated by networks of non-spiking interneurones in which slow oscillations in membrane potential (Pearson and Fourtner, 1975) can induce cyclical transmitter release which in turn induces rhythmic firing. Robertson (1986), in

discussing the generation of the locust flight rhythm, states that oscillations in membrane potential of both interneurones and motoneurones result from phasic excitatory and inhibitory synaptic input, and that there is no evidence that any part of the oscillation is endogenously generated.

Clearly, however, motoneurone D_f possesses slow regenerative properties which give it the ability to structure its own, and thus motor output. Plateau potentials offer an important synaptic amplification system: a brief synaptic input may be enhanced in both duration and intensity. Thus such events may be important in timing the final motor rhythm.

It is possible that plateau properties may only be apparent in D_f under certain circumstances. In decerebrate cats plateau potentials can be recorded from motoneurones receiving tonic afferent input; but in spinalized animal these events only occur after injection with serotonin and noradrenaline precursors (Conway *et al*, 1988; Hounsgaard *et al*, 1988). Similarly, endogenous oscillatory activity can only be recorded from locomotor motoneurones of the lamprey in the presence of an NMDA receptor agonist (Wallén and Grillner, 1987). Such modulation of plateau properties could be important in D_f: a modulator enabling plateau production by D_f could enhance the response to synaptic drive, thereby allowing the neurone to be recruited into the escape response under appropriate conditions.

Although this study has focused almost exclusively on D_f, there is some evidence to suggest that other insect motoneurones may possess similar membrane properties (Figs. 3.36, 3.38). Plateau properties could be particularly 'useful' to a neurone such as cell 3 which appears to be active during both walking and flight. There is evidence from other preparations that neurones can be switched from one circuit to another by 'on-off' modulation of plateau properties (Hooper and Moulins, 1989), and such a switching mechanism could determine which locomotor circuit cell 3 is recruited into. Plateau properties in the locust FETi could alter the sensitivity of this neurone to excitatory synaptic drive and be important in timing of the locust jump. However, at present, the spontaneous depolarising events recorded from FETi in the presence of PTX have not been demonstrated to result from effects of this agent upon intrinsic membrane properties alone. These events may result (entirely, or in part) from the unmasking of excitatory synaptic input to FETi by PTX, as a consequence of PTX-mediated blockade of inhibitory inputs to the cell.

CHAPTER 4. TIME-DEPENDENT EXCITABILITY CHANGES IN MOTONEURONE D_f.

INTRODUCTION

Many of the experiments which constitute the main body of this thesis were long-lasting (up to 4 hours). During the course of some of these experiments, the excitability of D_f appeared to change such that the cell, when polarised with brief depolarising current pulses, responded by generating one or more all-or-none action potentials which appear distinctly different from the attenuated axon spikes which often surmount the crest of plateau potentials.

Previous studies of the excitability of motoneurone D_f have concluded that its soma only produces action potentials after pharmacological, or other experimental intervention (Pitman *et al.* 1972b; Pitman, 1979; 1988). This contrasts with molluscan systems where most central neurone somata can produce all-or-none action potentials. This lack of impulse generating capability has suggested that insect motoneurone somata have no functional requirement to support action potentials in the normal state and, therefore, that soma-spikes play no role in information processing within these neurones (Pitman, Tweedle and Cohen, 1972b; Hoyle and Burrows, 1973; Gwilliam and Burrows, 1980).

This chapter focuses on time-dependent excitability changes in D_f in some detail, describing the time-dependent action potentials, locating their site of origin within the neurone and determining their ionic dependence. It also addresses the question of whether these excitability changes are associated with changes in the ability of this cell to produce plateau potentials: can plateaux and time-dependent action potentials co-exist in neurones?

RESULTS

4.1. Time-dependent action potentials in D_f

The response of D_f to short, 50ms depolarising pulses of small magnitude was essentially passive (Fig. 4.1Ai). In freshly dissected, recently impaled preparations the response to increased levels of current injection was a series of damped membrane oscillations (Pitman, 1979). The magnitude of these oscillations increased with the size of the current pulse injected (Fig. 4.1Aii,iii) (see also section 3.1.2.). Preparations demonstrating such responses were left in normal saline and the membrane response to current injection monitored over long time periods. With increasing time into experiments (between 1-4 hours, depending on the preparation), the response to brief depolarising pulses changed from graded membrane oscillations to an all-or-none action potential with a definite threshold (Fig.4. 1B). When longer

duration pulses were applied to the neurone a train of action potentials could be elicited, each with a similar amplitude and distinct afterhyperpolarisation (Fig. 4.1C . Increasing the current strength of such pulses had little effect on action potential amplitude.

Action potentials recorded in this way had a mean amplitude of 18.6mV (S.E. ± 0.7, n=18) when measured from the inflection on their rising phase (Inflection marked in Fig. 4.2A). Measurements of action potential amplitude were taken in this way to avoid problems which could arise measuring amplitude from the resting potential, V_r, (where changes in V_r could introduce false changes in amplitude), or from peak to trough of the action potential (where changes to the after-spike hyperpolarisation could similarly impose false changes in spike amplitude). In some cases the amplitude of spikes increased with time (Fig. 4.2) so attempts were made to standardise measurements: values for mean amplitude were taken from action potentials recorded immediately the spiking response was observed. Although action potential amplitude could increase with time, action potentials did not generally overshoot 0mV.

The duration of action potentials at their half-height was measured as 3.5ms (S.E. ± 0.1, n=18). Action potential duration was monitored throughout experiments; but, whilst spike amplitude could increase with time, spike-duration appeared to remain unchanged with time.

FIGURE 4.1: Development of all-or-none action potentials in the soma of D_f. A (i) Passive response to subthreshold depolarising pulse (4nA) applied to a freshly dissected recently penetrated neurone; (ii), (iii), damped membrane oscillations produced in response to 10nA and 16nA depolarising pulses respectively. (B) All-or-none action potential evoked by 8nA depolarising pulse two hours post impalement. (C) Train of action potentials evoked by 5nA depolarising pulse applied to a different preparation 1.5 hours after initial recording. Horizontal bars represent duration of injected current pulses. Resting potentials of cells (A,B) and (C) both -70mV.

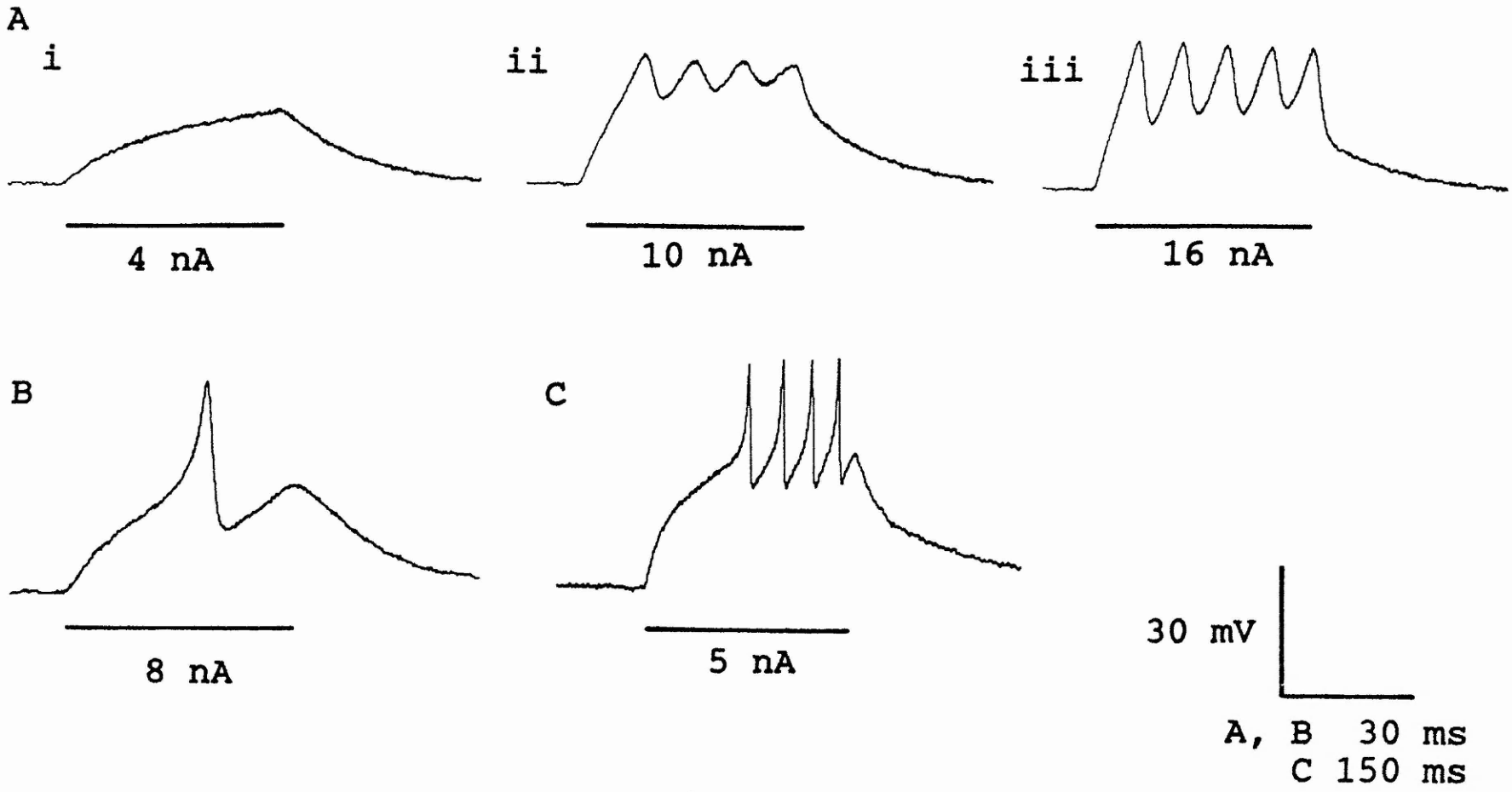
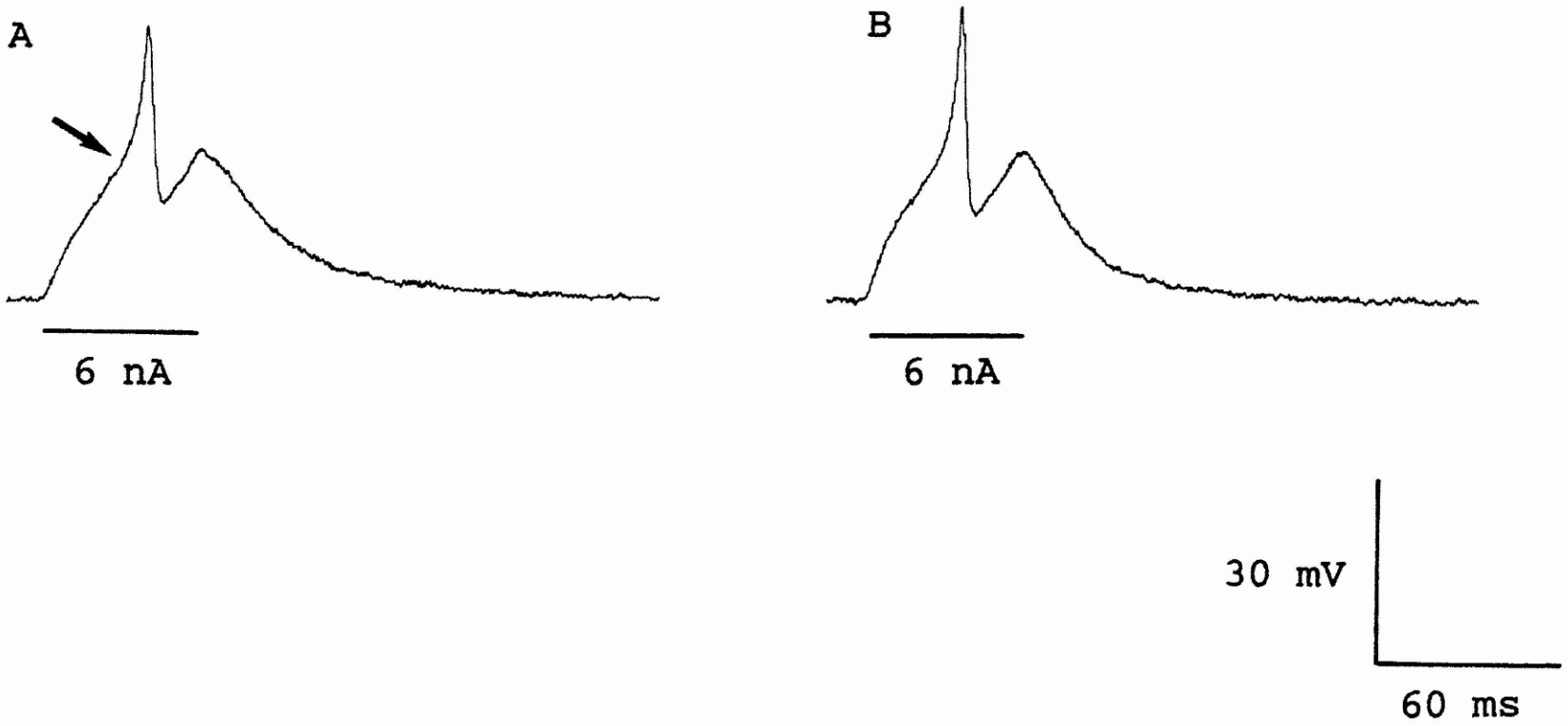

A
i
ii
iii
4 nA
10 nA
16 nA
B
C
8 nA
5 nA
30 mV
A, B 30 ms
C 150 ms

FIGURE 4.2: Action potentials recorded from the soma of a spiking neurone 1.5 hours (A) and 2.5 hours (B) after first recordings made. The amplitude of the action potential in (B) is clearly larger than that in (A) (26mV as opposed to 20mV). The arrow in (A) marks the inflection on the rising phase of the response. Resting potential of cell -70mV. Horizontal bars represent duration of depolarising current pulses.


A
6 nA
B
6 nA
30 mV
60 ms

The time-dependent increase in excitability did not appear to be a result of changes in the properties of D_f consequent upon damage resulting from impaling the neurone with microelectrodes. Some preparations were left for several hours prior to impalement; all-or-none action potentials could be recorded from these immediately following dual impalement, indicating that it is time after dissection which is the important parameter, as opposed to time after impalement.

4.2. Changes in effective membrane resistance and time constant

Measurements of effective membrane resistance and time constant were taken at increasing intervals during experiments. Changes in these parameters did not appear to correlate with changes in the excitability of the neurone. In some cells, for example, the conversion to spiking occurred without any change in input resistance; in others this parameter increased; whilst in a small number of experiments the input resistance appeared less after the onset of the spiking phenomenon. Similar observations were made with respect to membrane time constant. Both parameters have previously been reported to increase with pharmacologically induced excitability (Pitman, 1979). The lack of consistent changes in the present work, however, indicates that these parameters are not principal determinants of excitability in these experiments.

During the course of development of time-dependent action potentials there was no consistent alteration in the resting potential of neurones that could account for the change in excitability.

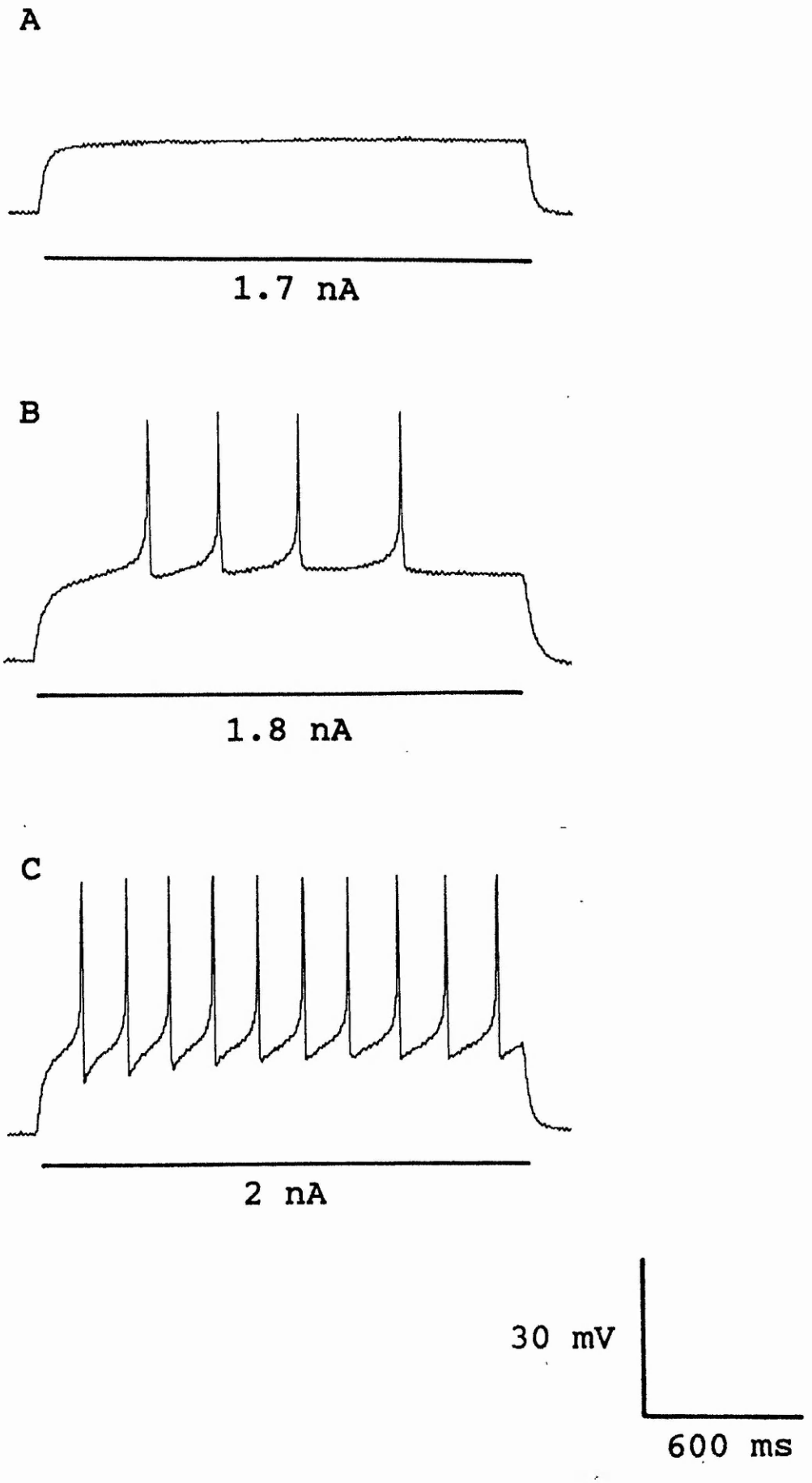
4.3. Topographical location of the spiking phenomenon

Experiments were performed to determine the site of origin of the action potentials generated following time-dependent changes in excitability. Long-term recordings were made from mechanically isolated somata to establish whether the action potentials recorded in the cell body result from changes in somatic membrane properties, or whether they reflect changes to some other part of the neurone.

Cells 'undercut' according to the methods described in chapter 2 were able to generate all-or-none action potentials under similar recording conditions to those for the intact neurone. Fig. 4.3 shows the response of a mechanically isolated cell body to current injection 1.5 hours post impalement. Injection of a relatively long duration current pulse of low magnitude produced a passive membrane depolarisation; larger levels of current were sufficient to elicit a train of action potentials. The inferences from this observation are twofold. Firstly, the changes in excitability were not associated with time-dependent changes to any direct synaptic modulation of the cell (the functionally isolated cell-body is separated from its synaptic inputs

which occur in the neuropile). Secondly action potential genesis appears to involve the neurone soma. However, the above observations do not preclude the possibility that other regions of the neurone (such as dendrites) also participate in generating time-dependent action potentials.

FIGURE 4.3: Recordings from a cell body mechanically isolated *in situ*. (A) Response to a relatively long duration, subthreshold (1.7nA) depolarising current pulse applied 1.5 hours after first recordings made. (B) A larger pulse (1.8nA) elicited a small train of action potentials. (C) A still larger depolarisation (2nA), applied ten minutes further into the experiment, significantly increased the spike frequency. Horizontal bars represent duration of depolarising current pulses. Resting potential -75mV.


A
1.7 nA
B
1.8 nA
C
2 nA
30 mV
600 ms

4.4. Ionic dependence of time-dependent action potentials

To determine the ionic basis of the time-dependent action potentials, preparations generating all-or-none action potentials were stimulated at regular intervals (between 0.6 Hz and 2.0 Hz) and superfused with saline containing tetrodotoxin, manganous chloride or cadmium chloride. After the effects of any of these agents had been observed, the preparation was washed with normal saline to achieve reversal of their action, where possible.

4.41. Tetrodotoxin

Tetrodotoxin was applied to preparations producing all-or-none action potentials (n=4). Fig. 4.4A,B shows that the amplitude of action potentials recorded from one of these preparations was not reduced by 6 minutes exposure to 10^{-6}M TTX; at this point the concentration of this agent was increased tenfold. This relatively high concentration of TTX also failed to reduce the amplitude of time-dependent action potentials in this preparation (Fig. 4.4C). For some preparations exposed to TTX, such as that used for Fig. 4.4, there was an increase in spike amplitude with time similar to that observed in cells not exposed to this agent. (The viability of the tetrodotoxin was tested on dorsal unpaired medial (DUM) neurones, which exhibit sodium-dependent spikes (Jėgo *et*

al, 1970); 10^{-5}M TTX abolished these spikes in under two minutes). TTX effectively abolishes the sodium-dependent spike in D_f induced following anoxia at a concentration of 2 x 10^{-7}M (Pitman 1988). It is unlikely, therefore, that any significant proportion of the inward current of the time-dependent spikes seen in the present experiments is carried by sodium ions.

4.42. Manganous chloride and Cadmium chloride

Bathing preparations in saline containing either 20mM or 40mM manganese ions (n=4) or 1mM cadmium ions (n=7) abolished the time-dependent action potentials within 5 minutes (Fig. 4.5); action potentials underwent a progressive decline before complete block. Increases in depolarising current injection (twofold or more) were unable to evoke action potentials in the presence of saline containing these ions, and so their effect was most certainly that of blockade, rather than a simple increase in threshold for the response. However, if the strength of the applied current was increased sufficiently, some preparations could give graded oscillations following treatment with either of these agents (Fig. 4.5B_{iii}); but such oscillatory responses differed markedly from all-or-none spikes. Block of spikes by Mn^{2+} or Cd^{2+} ions was at least partially reversible on washing preparations with normal saline. The above findings indicate that a large proportion, if not all, of the current carrying the time-dependent

spikes is carried by calcium ions. Often, the blockade of action potentials was accompanied by a steady membrane depolarisation, which was reversible on washing the preparation in normal saline. This effect is similar to that observed when investigating the ionic dependence of plateau potentials (section 3.3.1.) and could be interpreted in terms of indirect effects of Mn^{2+} and Cd^{2+} ions on the resting potential *via* the calcium-dependent potassium current (I_C) present in D_f (Thomas, 1984) (see also section 3.3.1B). However, the magnitude of this depolarisation (generally <10mV) was insufficient to cause spike block by inactivation of inward currents.

FIGURE 4.4: Effect of tetrodotoxin on time-dependent action potentials. (A) Action potential recorded in normal saline. (B) Action potential recorded following six minutes exposure to 10^{-6}M TTX. (C) Action potential recorded after 10 minutes exposure to 10^{-5}M TTX (total exposure 16 minutes). Horizontal bars represent duration of continuously applied current pulses (7nA, 0.8Hz). Resting potential -75mV.

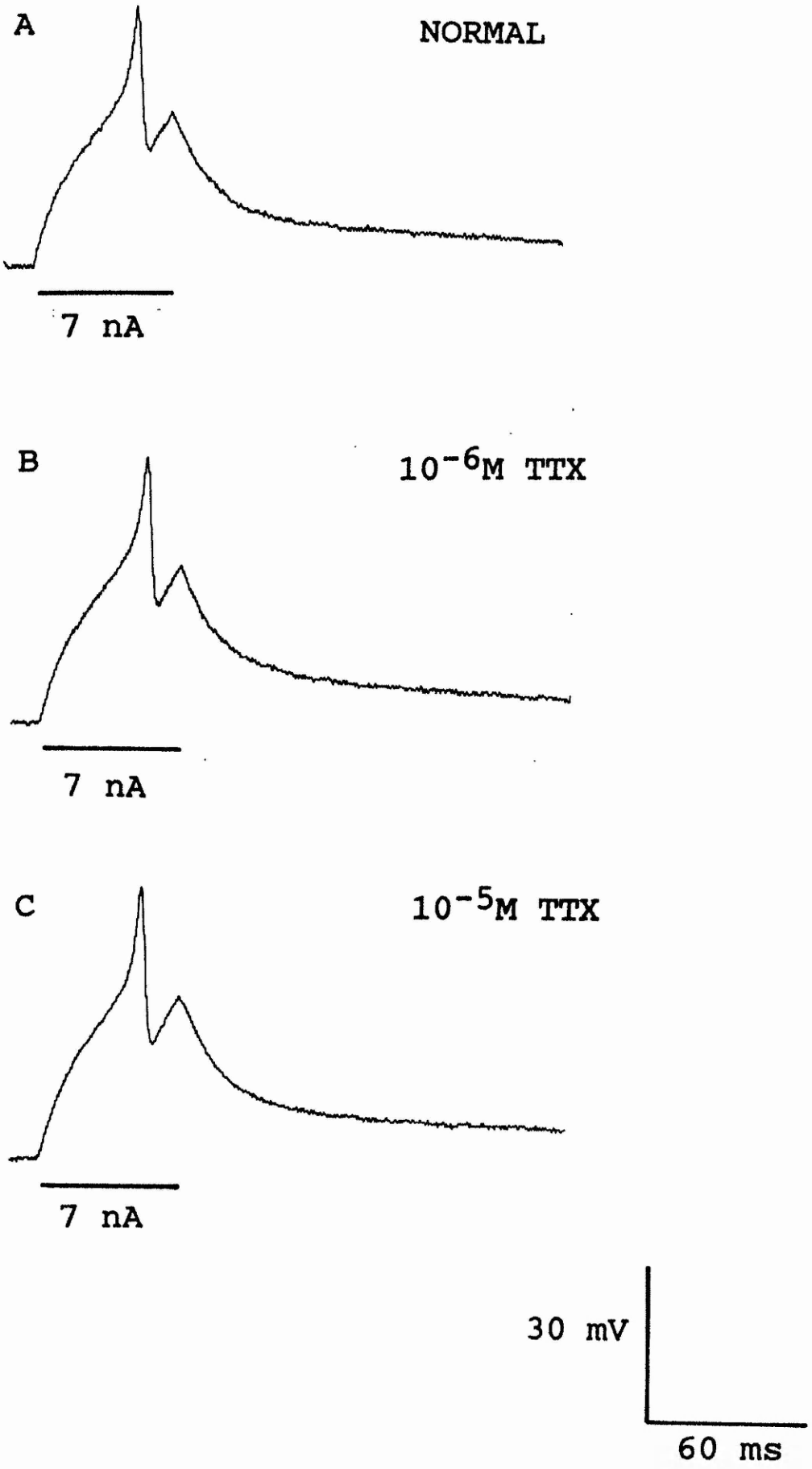
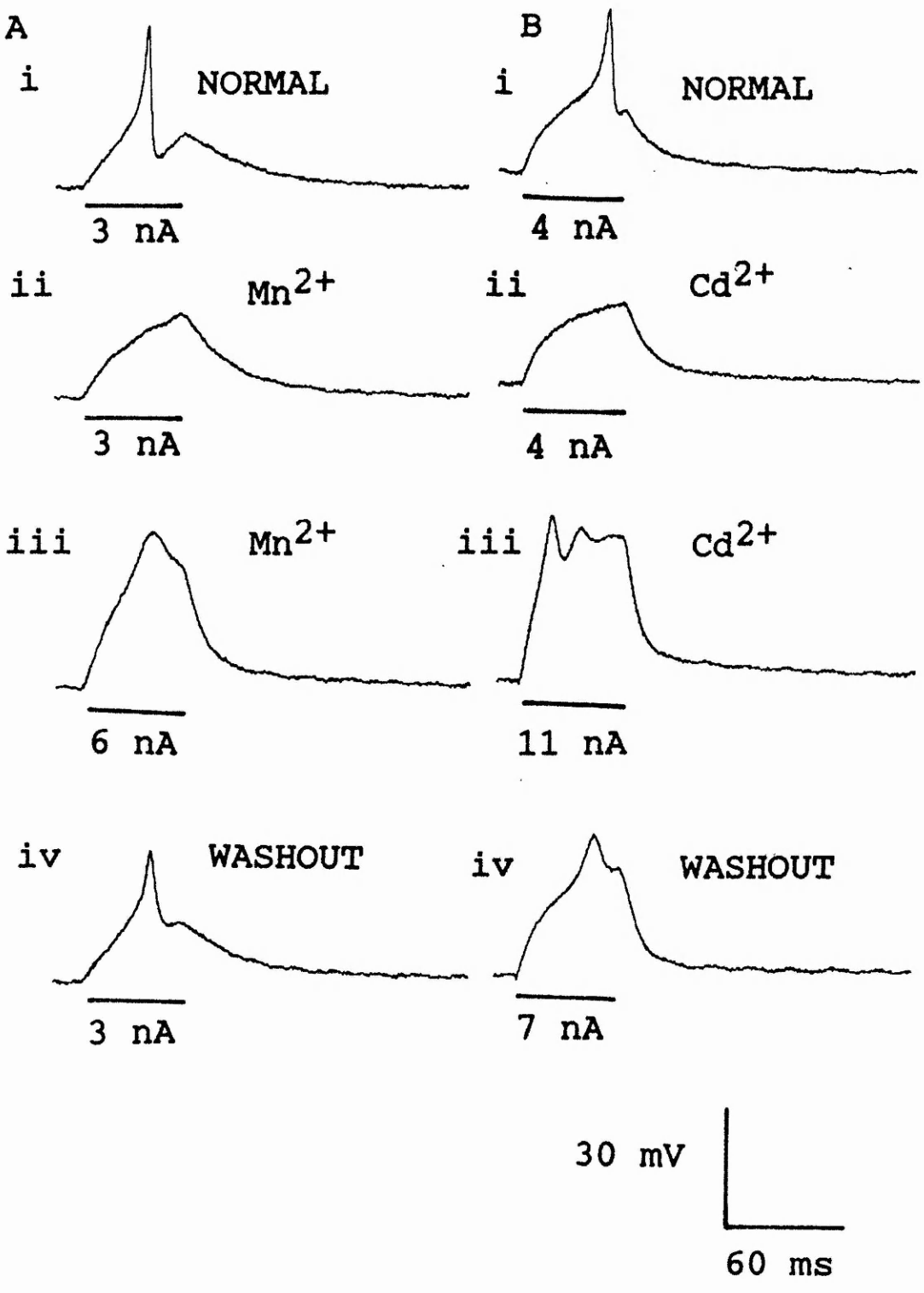

A
NORMAL
7 nA
B
10-6M TTX
7 nA
C
10-5M TTX
7 nA
30 mV
60 ms

FIGURE 4.5: (A) Effect of manganese ions (40mM) on time-dependent spikes. (Ai) Action potential recorded in normal saline. (Aii) Blockade of action potential within 30 seconds of manganese addition. (Aiii) A twofold increase in current injection failed to evoke an action potential two minutes after Mn^{2+} addition, although some graded response could be observed. (Aiv) Action potential recorded after 10 minutes washout in normal saline. Resting potential (i, ii), -67mV; (iii), -65mV; (iv), -67 mV.

(B) Effect of cadmium ions (1mM) on time-dependent spikes (different preparation from (A)). (Bi) Action potential recorded in normal saline (pulse amplitude 4nA). (Bii, iii) Responses to 4nA and 11nA, respectively, recorded 1 minute after addition of Cd^{2+}, demonstrating blockade of the action potential; some graded oscillatory response could be evoked by increased levels of current injection (Biii). (Biv) Limited recovery of response after 10 minutes washout in normal saline (pulse amplitude 7nA). Resting potential (i), -68mV; (ii, iii), -64mV; (iv), -65mV. In both A and B horizontal bars represent duration of depolarising current pulses (stimulus frequency 1 Hz).

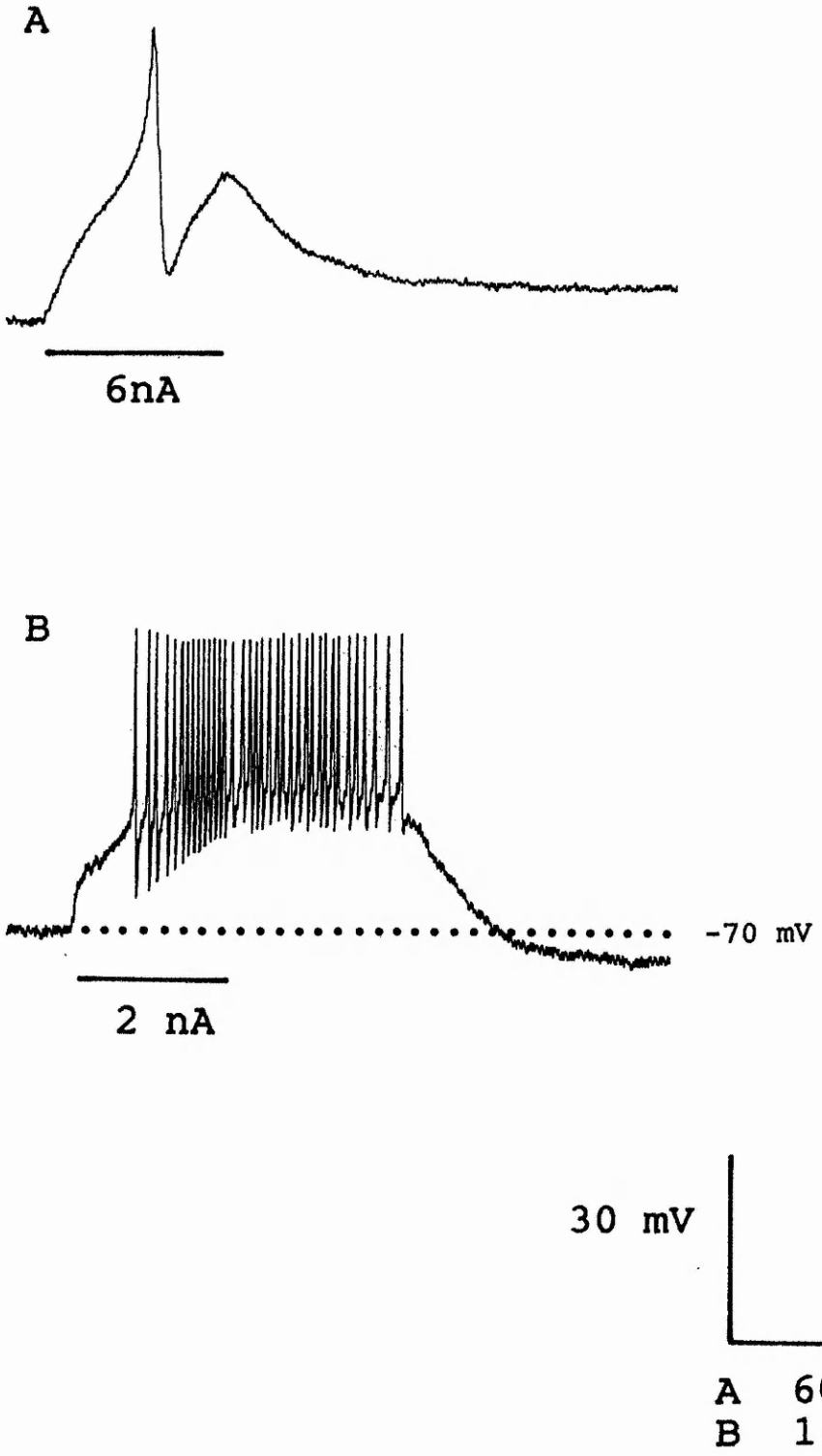

A
i
NORMAL
3 nA
ii
Mn2+
3 nA
iii
Mn2+
6 nA
iv
WASHOUT
3 nA
B
i
NORMAL
4 nA
ii
Cd2+
4 nA
iii
Cd2+
11 nA
iv
WASHOUT
7 nA
30 mV
60 ms

4.5. Do time-dependent changes in the excitability of D_f affect its ability to generate plateau potentials ?

An important question arising from the observation of time-dependent changes in the excitability of D_f is whether or not this cell retains the ability to generate plateau potentials once it has started to produce all-or-none spikes.

In both mechanically isolated somata and intact neurones, plateau potentials could be recorded throughout experiments in which the excitability changed such that the neurone generated all-or-none action potentials. Moreover, plateau potentials could be recorded synchronously with time-dependent spikes: plateau potentials induced bursts of these spikes which far outlived the applied depolarisation (Fig. 4.6). The fact that these two types of event can co-exist suggests that the calcium channels involved in the generation of plateau-responses differ from those carrying the inward current of the time-dependent action potential and, therefore, that the time-dependent action potentials do not result from changes to the channels involved in plateau potential generation (or *vice versa*). This observation is in accord with that made with respect to citrate-induced spikes and plateau potentials in chapter 3 (Fig. 3.25 to Fig. 3.30).

FIGURE 4.6: Responses of a neurone to short (A) and relatively long (B) duration suprathreshold depolarising pulses applied 2 hours after initial recordings made (current pulse magnitude 6nA and 2nA respectively). Whilst a relatively short (<60ms) pulse elicits a single action potential, a longer duration pulse (1.2s) produces a plateau potential outlasting the pulse which drives a burst of action potentials. Dotted line in (B) represents resting potential of cell; the plateau is followed by an afterhyperpolarisation. Horizontal bars represent durations of depolarising current pulses. Resting potential -70mV.


A
6nA
B
-70 mV
2 nA
30 mV
A 60 ms
B 1.5 s

DISCUSSION

The observation of time-dependent action potentials in motoneurone D_f raises a number of relevant questions. Firstly, can time-dependent action potentials be ascribed to changes to a healthy neurone, or are these events consequent upon cell damage and, therefore, related to the demise of the neurone?

It is unlikely that the observed changes in excitability are related to damage caused by impalement (or recovery from impalement), for, in some instances, action potentials could be recorded immediately following penetration. Therefore, it appears that it is time following dissection, rather than time following impalement which is the important variable determining the degree of excitability. Moreover, the development of enhanced excitability did not appear to correlate with a fall in resting membrane potential or effective membrane resistance which would be consistent with pathological changes to the neurone. Indeed, the values for both membrane resistance and membrane time-constant obtained in the current study are somewhat higher than those reported in an earlier examination of D_f (Pitman, 1979) and, in addition, all-or-none action potentials could be recorded from D_f for long periods (several hours) after their initial appearance. Thus, the available criteria provide no indication that the ability of the untreated D_f to generate calcium-

dependent action potentials relates to pathological changes to membrane properties of the neurone.

A second question relates to the mechanisms underlying the time-dependent changes in excitability observed in this series of experiments. Calcium-dependent action potentials have been recorded from the soma of D_f following extracellular TEA application, or the intracellular injection of citrate anion or the calcium chelator EGTA (Pitman, 1979); and these have been associated with changes in a tonically active outward conductance which is dependent on intracellular calcium ion concentration (Pitman, 1979). (Such a conductance (I_c) was subsequently identified, under voltage-clamp, for D_f and cell 3 (Thomas, 1984; Nightingale and Pitman, 1989).) It was proposed that changes to this conductance, resulting from manipulations of intracellular calcium concentration, could make cells more excitable by disturbing the balance between inward currents and outward currents in favour of inward currents; normally when the neurone was depolarised the potassium conductance became sufficiently large to shunt inward calcium currents, thereby preventing action potential genesis. Reduction of $[Ca^{2+}]_i$ would lower I_c, reduce the shunt on Ca currents and permit action potential production (Pitman, 1979). However, it is difficult to account for the changes in excitability reported here in terms of changes to a resting outward conductance since, whilst citrate- or EGTA-induced

spikes occur with an increase in effective membrane resistance (Pitman, 1979), time-dependent spikes appear independently of changes to this parameter. This difference, however, is attributable, at least in part, to the significantly more negative mean resting potential obtained in the present study (-70.8mV ± 1.0; mean ± S.E) compared to that reported by Pitman (1979) (-61.2mV ± 1.6; mean ± S.E.; n=5). The resting potential reported in the earlier study may have been sufficiently positive to activate an outward conductance that is absent at the resting potentials recorded here. One possible reason for the more negative values for membrane potential (and higher values for input resistance and time-constant) reported in the present study than those presented by Pitman (1979) is that the experimental chamber has been redesigned so that recirculation of the saline solution is considerably more efficient (see section 2.2.1). As discussed in the preceding chapter (section 3.14), this alteration to the experimental set-up may mean that it is now possible to observe membrane properties in D_f that were not apparent in early studies.

The observed changes in excitability may result from modulation of voltage-dependent ion-channels by some intercellular signalling molecule, particularly since the effects are both delayed (spikes are not observed in freshly dissected preparations) and long-lasting. Many ion channels are susceptible to modulation, both from

invertebrates and vertebrates (reviews by Levitan and Kaczmarek, 1987; Levitan, 1988; and by Koketsu, 1984); much of this modulation is exerted by neurotransmitters acting via a second messenger system. It is unlikely that modulation of the excitability of D_f can be ascribed to time-dependent changes to specific modulatory inputs to the cell, however, since time-dependent spikes can be recorded from neurone somata which have been isolated from their dendritic arbour, and thereby any direct synaptic input. Thus, any modulatory effect must result from substances released into the ganglionic environment surrounding D_f, possibly consequent upon surgical intervention; or, conversely, result from the removal of substances from that environment by the recirculating saline in the preparation bath. Although no direct evidence for such modulation has been obtained in the present study, there is evidence that action potentials from some insect cells (oocytes) are susceptible to modulation (O'Donnell and Singh, 1988).

Of the ion channels likely to be involved in switching from non-spiking to spiking modes, calcium and potassium channels offer the clearest targets for modulation. Any modulation of calcium channels involved in all-or-none spike generation that does occur, must do so independently of changes to slower channels carrying the current of plateau potentials, since the two events can co-exist. In addition, a modulated increase in inward calcium current must be coupled to suppression of

outward current: in the absence of suppressive modulation of outward currents any enhancement in calcium current will result in increased activation of I_c.

Potassium channel modulation, therefore, offers a particularly attractive mechanism by which excitability could be controlled. Previous experiments with TEA have demonstrated that suppression of potassium currents can enhance the excitability of D_f (Pitman, 1979). (Observations from a limited number of voltage-clamp experiments are consistent with a time-dependent decrease in voltage-dependent outward currents, since outward current responses generated by voltage-clamps appear to decrease with time (personal observations).)

Alternatively, modulation of the excitability of D_f could be brought about by changes in intracellular conditions. Such changes are involved in the increased excitability of crustacean muscles under conditions of anoxia or lowered pH; both treatments induce calcium-dependent action potentials in normally inexcitable cells, probably as a result of blockade of delayed potassium currents by hydrogen ions (Moody, 1978; 1980). It is unlikely that time-dependent action potentials in D_f are produced by a similar mechanism, since previous experiments have shown that lowering intracellular pH (by acid injection or external ammonium chloride application) reduces, rather than enhances the excitability of this cell (Pitman, 1988). However, since a fall in intracellular pH reduces the

excitability of D_f, it is possible that neurones undergo a fall in intracellular pH during dissection and isolation of the nerve cord and that the increase in excitability reflects a long-term recovery to normal pH. At present, the precise mechanisms underlying the time-dependent increase in the excitability of D_f remain unclear.

The final question which should be addressed concerns the implications of the current observations. Until now, insect motoneurone somata have been viewed as incapable of actively supporting action potentials in the normal state (Pitman, Tweedle and Cohen, 1972b; Hoyle and Burrows, 1973; Gwilliam and Burrows, 1980). That the soma of D_f can support time-dependent action potentials suggests that some insect motoneurone somata can participate in action potential generation, without any pharmacological, or other experimental manipulation of excitability. The ability of the soma to participate in the generation of calcium-dependent action potentials may itself have implications in terms of intracellular signal integration (this was first suggested in 1979 (Pitman)). However, at present these functional implications remain unknown.

CHAPTER 5. FUTURE DIRECTIONS AND CONCLUSIONS.

5.1. Thesis in context.

The results presented in this thesis contain two particularly interesting findings. Firstly, insect neurones are capable of generating plateau potentials which originate, at least in part, from the soma and which can drive bursts of axonal impulses. Secondly, insect motoneurone somata can generate action potentials in the absence of pharmacological or other experimental intervention. The ability of these two types of activity to co-exist in neurones (both are predominantly Ca-dependent) also provides the first evidence for different types of calcium channel in insect neurones.

One important conclusion from these observations is that it is no longer correct to describe insect motoneurone cell bodies as incapable of supporting regenerative activity in the absence of pharmacological or other experimental intervention (Pitman *at al*, 1972b; Pitman, 1975; Pitman, 1979; Gwilliam and Burrows,1980). In fact, the cell body of the untreated D_f can participate in the production of two distinctly different regenerative events.

A second conclusion from this study is that, since plateau potentials can drive bursts of axonal action potentials in an insect motoneurone, plateau

potentials could contribute to rhythmic pattern generation in these animals (this contrasts with: Robertson, 1986 and Robertson and Reye, 1988). Although this study does not show a specific behavioural role for the plateau potentials recorded from motoneurone D_f, it demonstrates that such events can (and probably do) enable individual motoneurones to structure their own output. Therefore, insects can no-longer be considered the major exception to the rule that rhythmic impulse generation depends upon a combination of synaptic connectivity and cellular properties of individual neurones (Russell and Hartline, 1978).

However, whilst motoneurones form the 'final common pathway' of networks generating locomotor activity (Kiehn, 1991) they are not usually part of the central pattern generating network which drives the underlying rhythm (Delcomyn, 1980). A crucial question, therefore, is whether or not neurones from insect central pattern generator circuits can exhibit plateau potentials. Can plateau potentials participate in generating the underlying locomotor rhythm, in addition to their role in structuring the form of the impulse-burst of individual motoneurones as in other preparations (Sillar and Elson, 1986; Wallen and Grillner, 1987; Kiehn, 1991)? This study sheds no light on this issue. However, Burrows, in a recent personal communication, suggested that activity has been observed in locust central interneurones

'which must be the result of plateau-type activity' (Laurent and Burrows, unpublished observations). It is likely, therefore, that in insects such membrane properties play a role in structuring centrally generated oscillatory rhythms.

The observation of time-dependent action potentials in D_f also has potential implications in terms of synaptic integration. It is possible that such action potentials could conduct regeneratively along dendritic processes in the cell and evoke transmitter release from output synapses of dendrites. Thus, the ability to switch on action potential generation in the cell could provide a second way of modulating and amplifying its output. If dendritic membrane can generate such action potentials, these events could also profoundly influence processing of incoming synaptic activity (Hounsgaard and Mitgaard, 1989).

The time-dependence of changes in the excitability of D_f is of particular significance for those using this neurone for pharmacological investigations: in many experiments using D_f there appears to be a shifting baseline of excitability (this ultimately leads to spike genesis). This could make interpretation of the effects of many pharmacological agents difficult, until such time as the mechanisms underlying the excitability change itself are understood.

5.2. Future directions.

"Philosophy, when superficially studied, excites doubt; when thoroughly explored, it dispels it." *Francis Bacon.*

Without doubt, this study has raised more questions than it has answered. Perhaps this is a good thing: there is evidently much scope for future work upon non-linear properties of insect neurones. Certain avenues of research must be explored in order to understand the functional significance of plateau potentials and time-dependent action potentials in the intact insect. These, and other relevant avenues of research, are discussed below.

5.2.1 Plateau potentials.

Three major questions must be addressed to place the observation of plateaux in D_f in a functional context:

(1) Can plateaux in D_f be evoked by stimulation of synaptic input? The answer to this question is crucial, since, for plateaux to play a functional role *in vivo*, these events must be controlled by synaptic stimulation (for examples: Sillar and Elson, 1986; Crone *et al*, 1988). This issue is not an easy one to address for D_f, as neurones presynaptic to this cell have not been identified. However, in recent experiments it has been possible achieve non-selective

synaptic stimulation of D_f by electrical stimulation of the contralateral nerve 5. The initial results of such experiments have proved promising: some cells under a depolarising bias can be made to produce plateaux in response to electrically evoked synaptic excitation; in other cells plateaux can be prematurely terminated, or their onset delayed by electrically evoked synaptic drive (Hancox and Pitman, unpublished observations; see Fig. 5.1). Thus, it appears that both excitatory and inhibitory synaptic inputs can affect plateau potentials in D_f. Further experiments must be performed to confirm these observations.

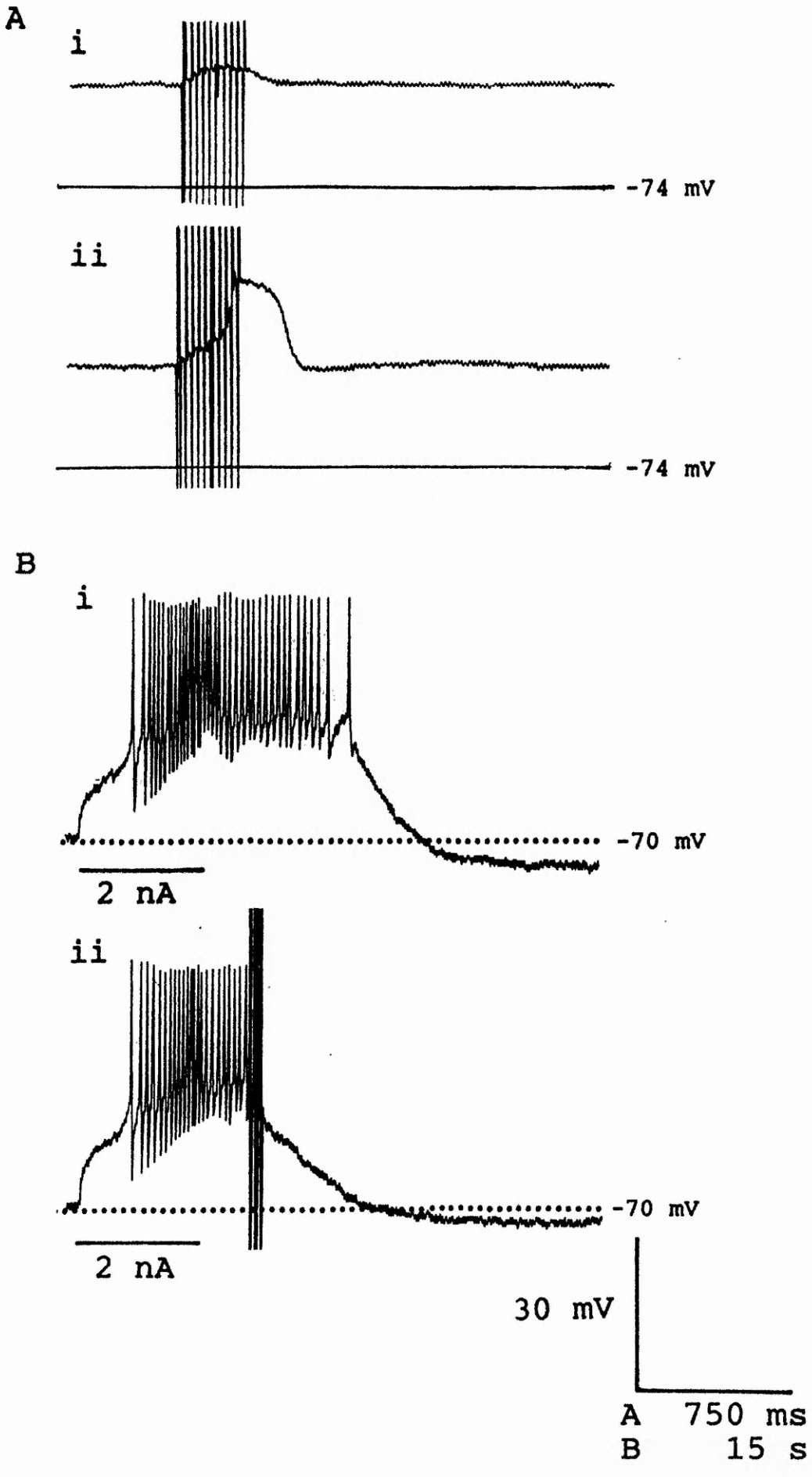
(2) How are plateaux modulated in D_f? Since there appears to be little difference in resting membrane properties between plateau-generating and non-generating neurones, some voltage-dependent modulation of plateau potentials must occur. It is important to demonstrate such modulation as this has behavioural implications: modulation could allow D_f to be recruited into different behavioural repertoires. Although some candidate modulatory compounds have been bath-applied, results have been equivocal. Candidate modulators for use in future experiments include: proctolin, octopamine, ACh, dopamine, serotonin, glutamate and FMRFamide. Recent experiments suggest that the ability of D_f to express plateaux may be enhanced by FMRFamide (Pitman and Hancox, personal observations).

FIGURE 5.1: Production (A) and premature termination (B) of plateau potentials in D_f by electrically evoked synaptic stimulation.

(Ai) Upper trace: Post-synaptic response of a cell receiving a sustained depolarisation (2nA) to a train of stimuli (7V, <1ms) applied to the contralateral nerve 5. Response represents summated EPSPs. Lower trace marks resting potential of cell (-74mV). (Aii) Response of same cell to a series of 8V, <1ms stimuli applied to the contralateral N5; the resulting excitatory post-synaptic response was large enough to evoke a plateau potential.

(Bi) Plateau potential, with superimposed time-dependent spikes, recorded from a different preparation in response to depolarising current injection. (Bii) Premature termination of plateau potential and spike burst by inhibitory synaptic input activated by stimulation of contralateral nerve 5 (a series of 10V, <1ms pulses was applied). Horizontal bars represent duration of applied current pulses. Cell resting potential -70mV.

Vertical lines in (Ai-ii) and (Bii) are the result of stimulus artefacts resulting from extracellular stimulation of nerve 5.


A
i
-74 mV
ii
-74 mV
B
i
-70 mV
2 nA
ii
-70 mV
2 nA
30 mV
A 750 ms
B 15 s

(3) How widespread amongst insect neurones is the ability to express plateaux potentials? There is some evidence that other 'fast' insect motoneurones, both from the cockroach and locust, can produce plateau-type activity (Figs. 3.36, 3.38). There are two reasons why some attention should, perhaps, focus on cell 3. Firstly, this cell appears to possess similar membrane properties to D_f (Nightingale and Pitman, 1989); secondly, the bifunctional nature of this cell means that modulation of plateaux could play a role in switching between output patterns appropriate for flight and walking.

Comparison between the properties of 'fast' and 'slow' motoneurones could prove interesting. Since the 'slow' coxal depressor motoneurone D_s appears to be dominant during normal walking (Pearson and Iles, 1970) its membrane properties should receive specific attention. A number of experiments performed upon the intact D_s and isolated D_s-soma, suggest that, unlike D_f, this cell does not produce plateau potentials. However, this cell appears to possess an active depolarising membrane response which is activated from relatively hyperpolarised membrane potentials (Pitman and Hancox, personal observations). This hyperpolarisation-activated depolarising response can be recorded from isolated somata, correlates with an inward current (under voltage-clamp) and affects the frequency of impulse generation in intact cells. Inward currents activated by depolarisations from

relatively negative membrane potentials play important roles in the oscillatory properties of inferior olivary neurones (see Llinas, 1989) and mediate changes between tonic and phasic firing in thalamic neurones (Suzuki and Rogawski, 1989); whilst post-inhibitory rebound excitation could play a role in sustaining motor rhythm production in the spinal cord from *Xenopus* embryos (Roberts and Tunstall, 1990). Therefore, a current activated from relatively hyperpolarised membrane potentials could have profound functional significance to a locomotor motoneurone such as D_s and requires in-depth investigation.

There is also considerable scope for investigating the ionic basis of plateau potentials in D_f. The use of ion-sensitive microelectrodes and ion-sensitive dyes such as Fluo-3 (for calcium) and SBFI (for sodium) should resolve any difficulties in data interpretation which can result from ion substitution experiments. Moreover, the use of ion-sensitive dyes, in conjunction with sophisticated microscopical techniques, could provide valuable information as to the regional location of ion channels within D_f. Such information could greatly facilitate future patch-clamp analysis of ion channel function in this cell, particularly if channel 'hot-spots' occur in certain regions of the cell membrane.

The isolated D_f-soma preparation offers an ideal preparation with which to investigate the nature of

the currents underlying plateaux. It affords similar advantages to the isolated cardiac motoneurone soma preparation of Tazaki and Cooke (1983c; 1986; 1990): such a preparation lends itself to voltage-clamp, since isolated somata do not present the same obstacles to 'space-clamping' that intact neurones from many preparations do. Calcium currents involved in plateau potential generation could be isolated in voltage-clamp experiments by perfusing preparations with a cocktail containing TTX (to block sodium currents) and TEA/4AP (to block potassium currents). Different organic and inorganic calcium channel blockers could be applied to determine whether the calcium channels involved in plateaux resemble one or other class of mammalian Ca-channel (see Triggle (1989) for a brief review). These channels could also be compared with those responsible for time-dependent action potentials.

The D_f preparation may provide an interesting model with which to investigate the effects of pathological intervention upon plateau potential production in motoneurones. Since these events can influence motor output profoundly (for reviews see Benson and Adams, 1989; Hartline *et al*, 1988; Kiehn, 1991) pathological interventions such as anoxia and physical damage (compression and partial axotomy) could influence motor output. D_f offers a particularly apt model to investigate the effects of anoxia and axotomy on

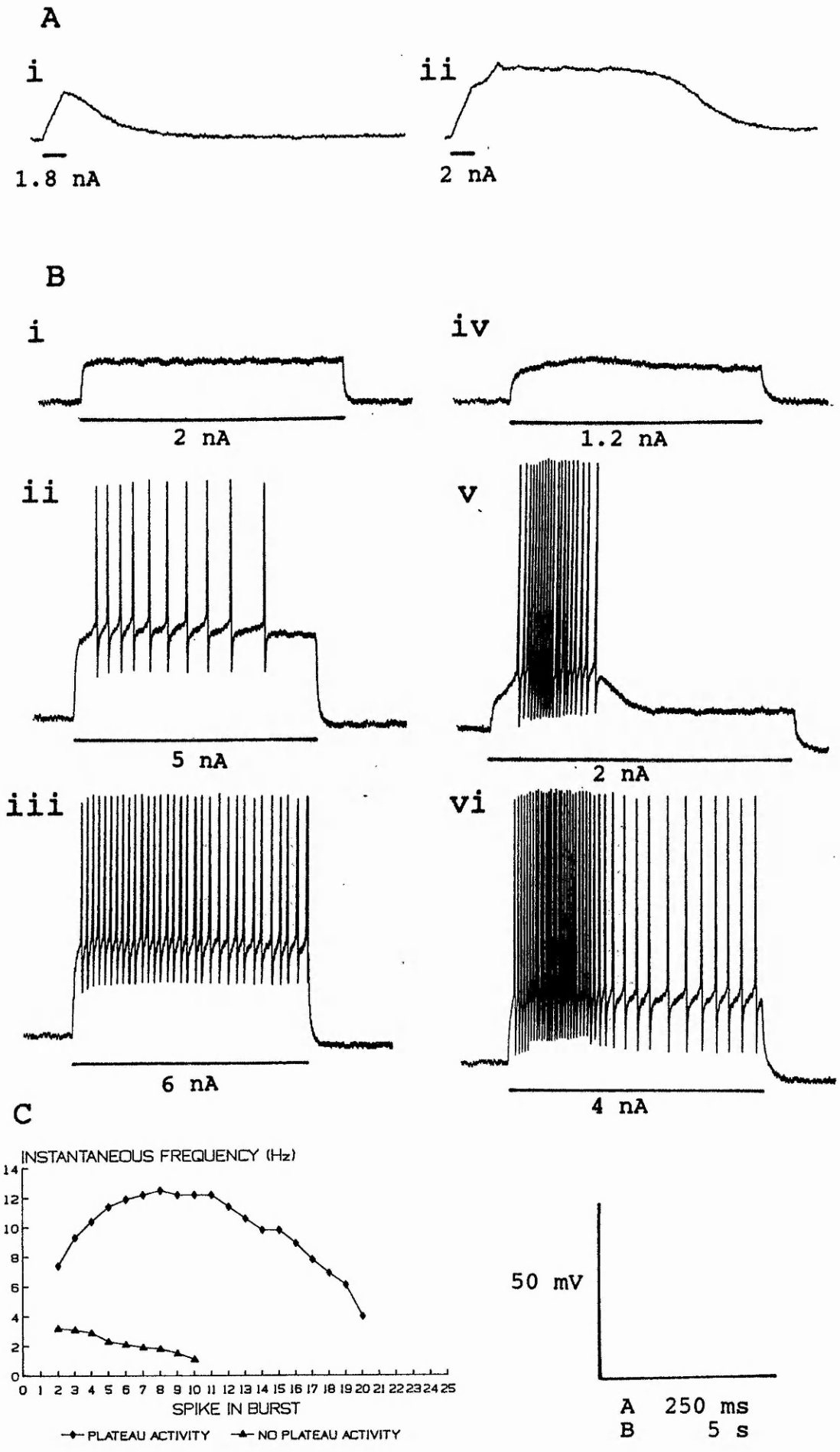
plateau production since it is already known that these treatments affect the excitability of the neurone (Pitman *et al*, 1972b; Pitman, 1975; Pitman, 1988; review by Pitman, 1990). Hypoxia has been shown to increase the amplitude of the bursting pacemaker potential in *Aplysia* R_{15} (Coyer, 1986), and preliminary experiments suggest that anoxia enhances plateau generating mechanisms in D_f (Hancox, personal observations): cells from animals which have been made 'partially' hypoxic (i.e. the cells do not produce the sodium spikes reported by Pitman, 1988) can respond to relatively short duration current pulses with plateaux (see Fig. 5.2A). Some cells generating sodium-spikes after anoxia display an action potential burst which appears to have an underlying slow active component (others do not); therefore plateaux may persist in cells which generate somatic action potentials following pathological intervention (see Fig. 5.2B). Further investigations may reveal how, if at all, the timing and/or phasing of the action potential burst is affected by anoxia (or partial axotomy) and be useful in determining how cell damage affects motor function.

FIGURE 5.2: (A) Records from a cell taken 24hrs after animal had received a 2hr period of anoxia. (Ai) Subthreshold response to brief depolarising current pulse. (Aii) A larger amplitude pulse elicited a plateau potential. This cell did not generate all-or-none action potentials.

(B) Responses of two cells from different animals (each exposed to 2hrs anoxia 48 hrs prior to recording) to long duration depolarising current pulses of increasing magnitude. (Bi-iii) One cell responded to suprathreshold pulses with trains of spikes, the frequency of which increased with the magnitude of the applied depolarisation. (Biv-vi) The second cell responded to a suprathreshold depolarisation with an intense burst of action potentials superimposed on a slower underlying depolarising event (Bv); larger amplitude depolarisations produced sustained firing of action potentials, with an intense burst of activity during the early part of the response (Bvi)

(C) Analysis of structure of spike-bursts in (Bii) (triangles) and (Bv) (diamonds). The relationship for the spike-burst in (Bv) resembles that for axonal impulse-bursts driven by plateaux in untreated neurones (see Fig. 3.8); that for (Bii) does not.

Horizontal bars represent durations of applied current pulses. Resting potentials (A) -67mV; (B) -70mV; (C) -70mV.


A
i
1.8 nA
ii
2 nA
B
i
2 nA
iv
1.2 nA
ii
5 nA
v
2 nA
iii
6 nA
vi
4 nA
C
INSTANTANEOUS FREQUENCY (Hz)
SPIKE IN BURST
PLATEAU ACTIVITY
NO PLATEAU ACTIVITY
50 mV
A 250 ms
B 5 s

5.2.2. Time-dependent action potentials.

There is considerable dimension for further work upon this phenomenon. Initial experiments could involve voltage-clamping time-dependent spikes and examining the voltage-dependence of the inward current which underlies them. Since time-dependent spikes can co-exist with plateau potentials, it would be desirable to try to differentiate between the calcium channels involved in the two events using organic/inorganic Ca-channel agonists/antagonists. It would also be useful to determine whether these calcium-dependent spikes are carried by an inward current with a similar voltage-dependence to that which underlies citrate-spikes in D_f, or whether there are three separate classes of Ca-channel in this neurone.

It is of most significance, however, to investigate the mechanisms by which the excitability of D_f changes during experiments. If excitability is enhanced by long-term changes in calcium concentration which only affect I_c (or any other calcium-activated outward conductance which may be present) at membrane potentials somewhat positive to rest, then this could be revealed by the use of Ca-sensitive dyes.

The role of candidate modulatory compounds in mediating changes in excitability could be investigated by ionophoretic/pressure injection of neurotransmitters. To investigate the role of possible metabolic changes, the role of preparation-

oxygenation could be elucidated by switching the source of recirculation from oxygen to nitrogen, whilst a role for hypoxia could be investigated by application of metabolic inhibitors such as such as cyanide (which induces hypoxia). The use of pH-sensitive electrodes could determine whether changes in intracellular pH play a role in the generation of time-dependent action potentials.

The third aspect of time-dependent spike generation that merits attention concerns a possible functional role for such spikes. An essential first stage in this research is to make simultaneous intra- and extra-cellular recordings to ascertain whether time-dependent action potentials can evoke activity in the axon. If so, it is likely that these events can influence the pattern of impulse generation by the axon, and, thereby, the output of the cell.

5.3. Conclusion.

Previously, insect neurones have been considered to be of interest because the neuronal circuits underlying flight and walking have lent themselves to study and because insect neurones have offered useful models for the pharmacological investigation of neurotransmitter action. However, the excitable properties of untreated insect motoneurone somata have not received significant attention, as these cell bodies have only appeared to exhibit regenerative activity following

cell-damage or pharmacological manipulation. This study shows that the intrinsic membrane properties of insect neurones must receive specific attention if rhythmic pattern generation, or pharmacological responses are to be fully understood. In a recent paper Laurent (1990) has reported that voltage-dependent outward rectification in local interneurones from *Schistocerca* may have functional relevance to synaptic transfer (EPSPs are much smaller at relatively depolarised membrane potentials). Plateau potentials and time-dependent action potentials are, perhaps, far more spectacular examples of non-linear properties of insect neurones. The scope for future research is vast (as discussed above), not least because such properties constitute a relatively new field of research in insect neuroscience. In conclusion, future research promises to demonstrate that non-linear membrane properties play far more important roles in determining patterns of activity in the insect nervous system than has been realized prior to this study.

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APPENDIX I. COMPOSITION OF INSECT SALINES.

SALINE	4M NaCl (ml)	1M KCl (ml)	1M $CaCl_2$ (ml)
Locust (normal)	37.5	5	4
Cockroach (normal)	53.5	3.1	9
Na-Free	*1	3.1	9
Low sodium (54mM)	(	*2	)

*1 Two alternate recipes were used for sodium-free saline. In one NaCl was replaced by 248.4 ml of 1M TRIS-HCl; in the other NaCl was replaced iso-osmotically with sucrose.
*2 Low-sodium saline was made by mixing normal saline with sodium-free saline in the proportions 1 : 3.

Stock solutions were made up to approximately 900ml in deionised, double-distilled water (Milli Q) before pH buffer was added. In standard cockroach and locust salines TES (N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid) buffer (Sigma) was added (2.292g/L: 10mM). This solution was acidic and was adjusted to pH 7.2 by adding sodium hydroxide whilst continuously monitoring the pH using a temperature-compensated pH meter (Pye-Unicam 292). In sodium-free salines TRIS (Tris[hydroxymethyl]aminomethane) buffer (Sigma) was added (1.211g/L: 10mM), producing an alkaline solution; in this case the pH was buffered using hydrochloric acid. In each case solutions were made up to their final volume (1000ml) and the pH rechecked.
Sodium and potassium concentrations were routinely checked using a flame-photometer (Corning).

APPENDIX II.

TOLUIDINE BLUE STAINING OF NERVE CELL BODIES IN INTACT GANGLIA (Modified from Altman, 1980).

Dissection.

Dissect the intact nerve cord out of the animal according to the methods outlined in Chapter 2. Ganglia of interest need not be desheathed for toluidine blue staining.

Stain

1g toluidine blue
6g borax
1g boric acid
100ml distilled water

pH: between 7.6-9.0

Immerse freshly dissected ganglia are in stain for 5 to 20 minutes in an oven at 50°C. The optimum duration of staining varies with temperature, age of stain, and preparation; if overstaining occurs it will be impossible to differentiate completely.

Differentiator and Fixative

Bodian's No.2 fixative

5ml formalin
5ml glacial acetic acid
90ml 80% ethanol

Transfer ganglia directly from stain to fixative, which must be changed several times in the first few minutes, as it becomes rapidly saturated with stain. Differentiation continues until cell bodies are visible under the dissecting microscope; other regions (neuropile and nerve roots) should be almost white (15-30 minutes). Since some stain is lost during dehydration, differentiation should be stopped just before the required contrast is attained.

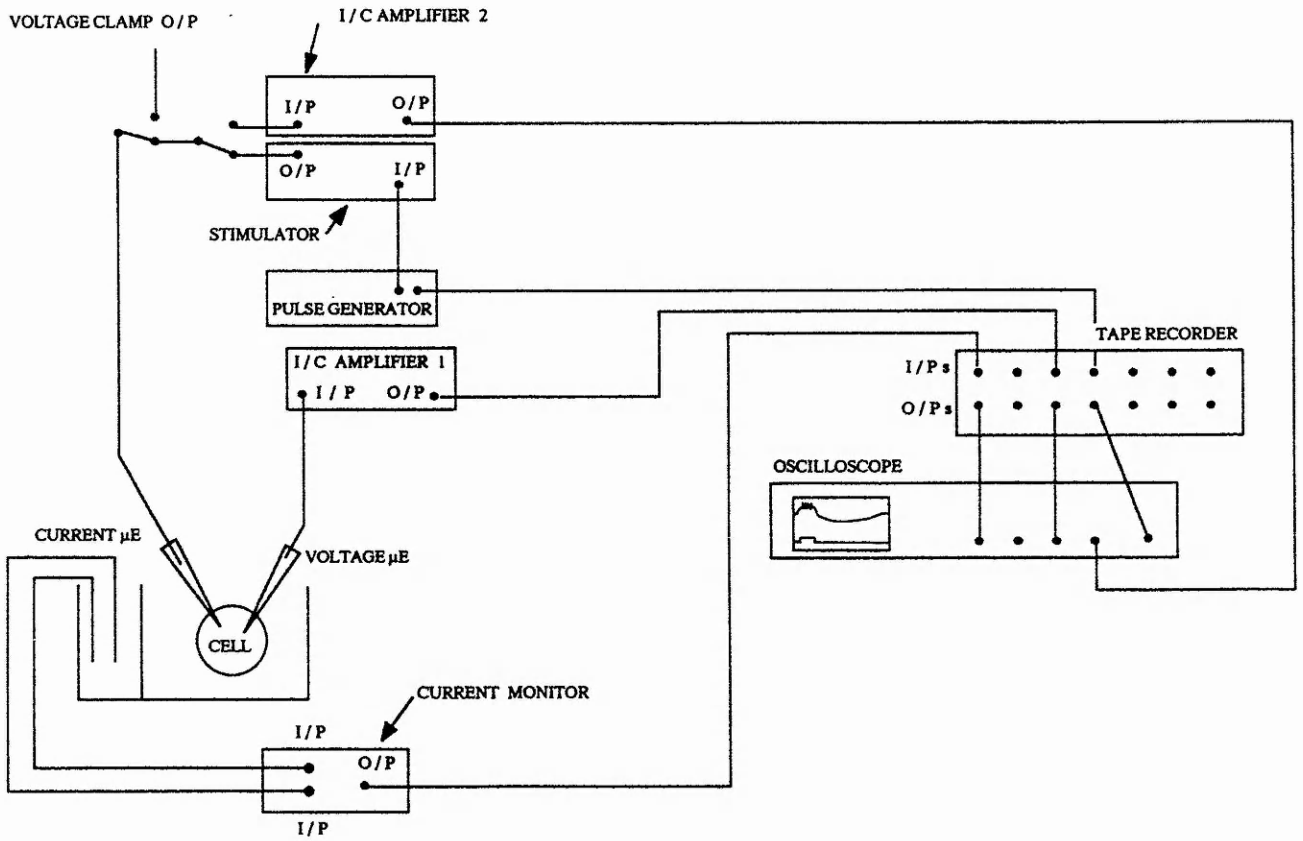
Dehydration

90% ethanol 5 minutes (X2)
100% ethanol 10 minutes (X2)

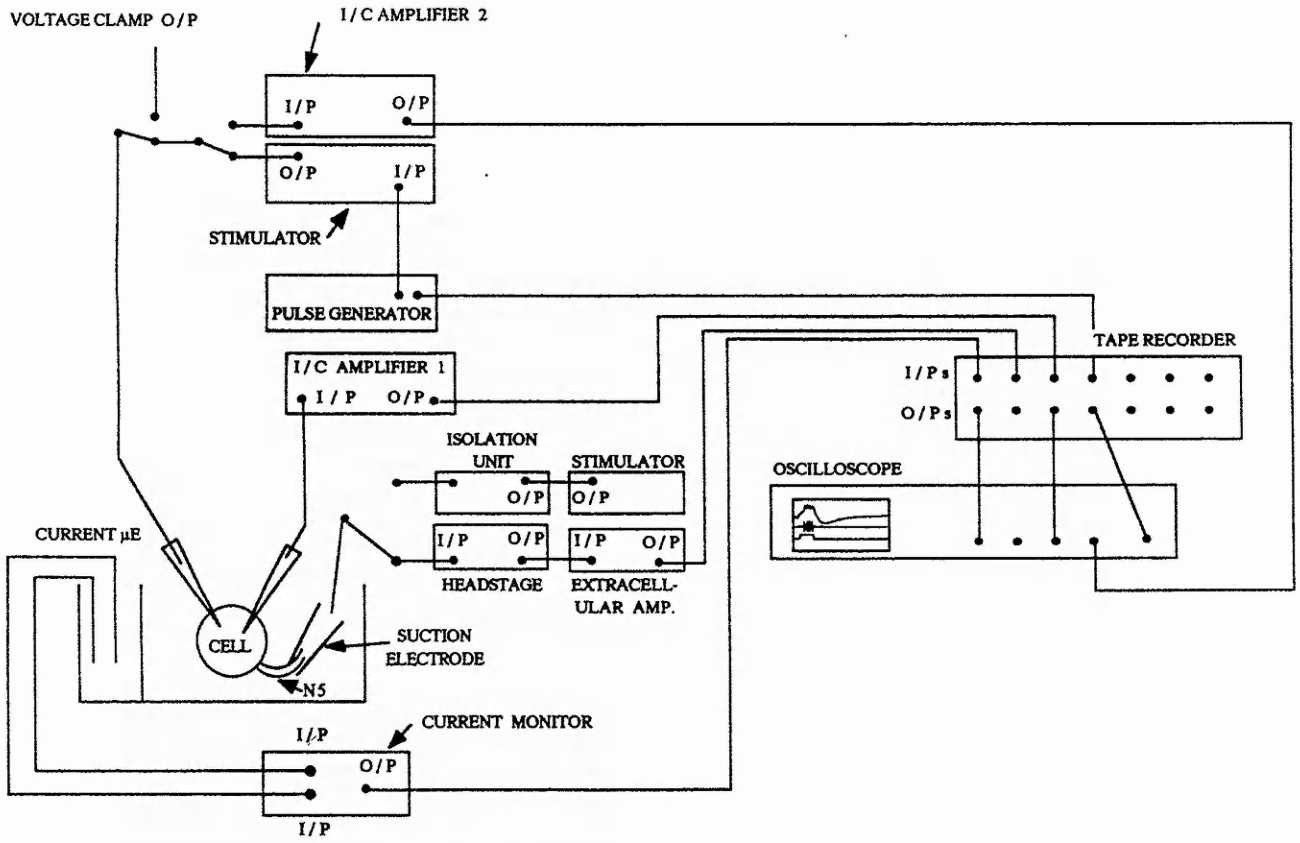
Clearing: Histoclear

Mounting: DPX

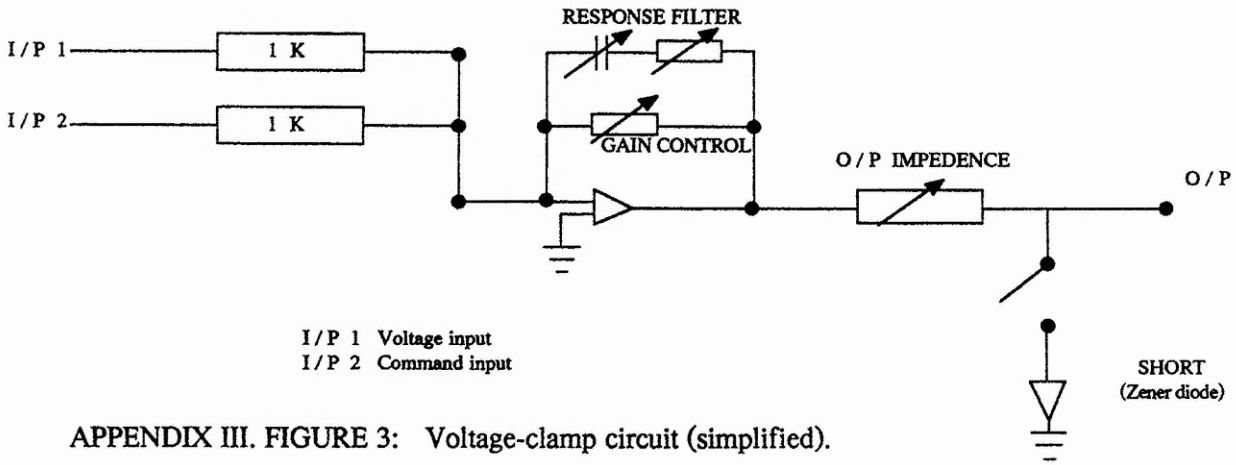
APPENDIX. III. ELECTRONIC BLOCK AND CIRCUIT DIAGRAMS.



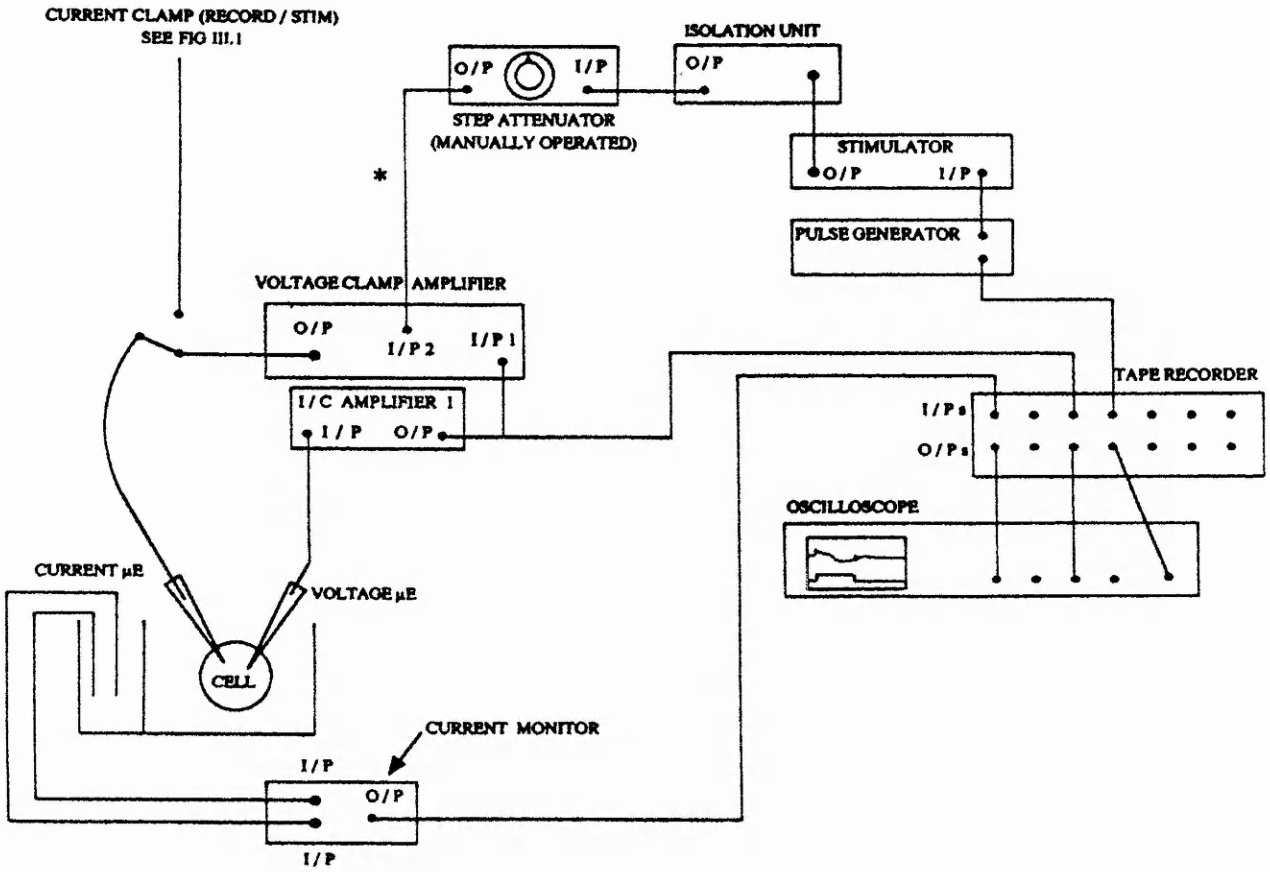
APPENDIX III. FIGURE 1: Block diagram respresenting recording configuration under current-clamp.



APPENDIX III. FIGURE 2: Block diagram of recording apparatus for concurrent intracellular recording and extracellular recording/stimulation.



APPENDIX III. FIGURE 3: Voltage-clamp circuit (simplified).



APPENDIX III. FIGURE 4: Block diagram representing voltage-clamp recording configuration. * The command input to the voltage-clamp amplifier could be manually operated (as shown), or operated by BBC series B (or IBM-compatable) software and appropriate interfaces (not shown).

APPENDIX IV

PROCESSING PHOTOGRAPHIC FILM AND PAPER.

A. **Kodalith Ortho Film Type 3.**

PQ Universal (Ilford)	1+9	variable
Stop bath (Kodak)	1+36	10secs.
Hypam Fixative (Ilford)	1+4	3-5mins.

(At 20°C, under red safelight)

B. **Multigrade Paper (II).**

Multigrade developer (Ilford)	1+9	1min.
Stop bath	1+36	10secs.
Hypam Fixative	1+9	3-5mins.

(At 20°C, under amber safelight)